

**EVALUATION OF THE PROTECTIVE EFFICACY OF HIV-1 PROTEASE
INHIBITORS AGAINST ENTEROVIRUS 71 INFECTION IN VITRO**

by

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A thesis submitted to Johns Hopkins University in conformity with the requirements for
the degree of Master of Science

Baltimore, Maryland

April 2014

ABSTRACT

Enterovirus associated hand, foot and mouth disease continues to be a prominent public health issue despite the numerous vaccine trials and drug development attempts. The lack of a specific and protective antiviral drug has made disease management extremely challenging and gravely underscores the need for effective therapy options. Nelfinavir, belonging to the HIV-1 protease inhibitors, was found to have significant antiviral activity compared to other HIV-1 protease inhibitors. With a low cytotoxicity and high inhibitory action against both the BrCr and H strain of EV71, nelfinavir exhibited potential broad spectrum antiviral properties. Additionally, its ability to impede the virus irrespective of the stage of the infection makes it a more promising therapeutic option. The data strongly support the potential usefulness of nelfinavir as a broad spectrum antiviral drug with significant protective efficacy against EV71 infection in vitro.

Acknowledgments

I would like to thank Dr. Xiao-Fang Yu and Dr. Richard Markham for giving me the opportunity to work on this project. Dr. Markham has been every kind and I am grateful for his comments and encouragement. I would like to thank Dr. Jay Bream for being a great advisor and for helping me whenever I reached out to him for guidance. Also, I would like to express my gratitude to Dr. Gary Ketner for being such an approachable advisor and for his input on my thesis. I am especially grateful to Dr. Xue Han for teaching me several techniques in the lab. She has always been very patient with all my questions and helpful. I must thank Dr. Wei Wei, Dr. Hoaran Gao and Dr. Xiaohong Zhou for being such good lab mates and readily helping me out with my experiments. Qimeng Gao and Dr. Sean Evans have been great company to hang out in lab and I will remember our little discussions in between incubation times. I cannot thank Snow Hou enough for being such a great friend and support system and for always being there for me no matter what. I could not have completed my thesis work without the support of my ScM friends. They have always managed to make me smile and laugh even during the toughest of times. I will always cherish all the moments I have had with them.

I have to thank Gail O'Connor without whom I would not have finished this project. She is an incredible person who can solve your problems in a jiffy. Lastly, I would like to acknowledge my PI, Dr. Xiao-Fang Yu for his expert guidance, and his help in the planning, execution and troubleshooting of my experiments. He has always encouraged me to work hard and learn new techniques. He has been a great mentor and I would like to thank him for all his encouragement, assistance and advice.

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INTRODUCTION

Hand, Foot and Mouth Disease & Enterovirus 71

Even after almost five decades since its discovery in 1969, hand, foot and mouth disease (HFMD) caused by Enterovirus 71 (EV71), continues to be a major global public health concern. Although the first case was identified in the United States, the incidence of the disease was later recorded to be predominant across the Asia-Pacific region at epidemic scales [1]. In a span of 4 years, 7.2 million probable cases and 267,942 deaths were reported in China alone [2]. Other countries that have been swept by the HFMD epidemic are Taiwan, Japan, Thailand, Singapore and Vietnam [3,4,5,6,7]. It is mainly a pediatric infection that is most common in children under 5 years of age, however, cases of infection in adults have also been recorded [8].

The disease is usually mild, self-limiting in nature and is resolved quickly in healthy children with a robust immune system. In a few cases where the infection is not cleared, children develop symptoms as the disease progresses. At the end of the incubation period, children present with fever and a papulovesicular rash on their palms, soles and in the oral cavity. In a lot of cases, the rash is accompanied by *herpangina* or painful sores and ulcers on the tongue and palette and upper respiratory tract infection [9]. For instance, in their three and half year long study in Malaysia, Mong How Ooi and co workers studied 725 children with HFMD, and noted 48% (169/333) of the patients who had skin vesicles positive for HFDM. The study further suggested that the use of throat swabs in addition to skin vesicle swabs increased the diagnostic value for HFMD (67% virus identification) [10]. Similarly in another study, Umesh Parashar and colleagues observed that 83% of

the 29 cases had either vesicular rashes or oral ulcers [11]. In the absence of supportive care, the infection spreads systemically and results in severe neurological sequelae, such as aseptic meningitis, encephalitis and flaccid paralysis [9]. The complications are a major challenge not only in terms of clinical management but also for diagnosis as they are common to a wide range of viral infections, including measles, rubella, mumps and adenoviruses [9]. This can cause incorrect diagnosis and subsequent initiation of therapy to resolve the symptoms. For instance, poor differentiation of HFMD from measles or adenovirus infection may result in unnecessary initiation of post exposure prophylaxis (Vitamin A treatment or passive immunization depending on the time of measles exposure or Cidofovir in rare cases of adenovirus infection). This delay in turn would result in a poor prognosis [12, 13].

Throat, ulcer and rectal swabs, vesicular fluid, CSF, serum and urine are usually collected for virus isolation and confirmation. Laboratory identification is done through cell culture using rhabdomyosarcoma cells, African green monkey kidney cells or human lung fibroblast cells. Serology and more recently molecular serotyping using PCR are being performed for a more definitive diagnosis [9].

In terms of prevention, although there is no licensed vaccine available for EV71 infection, fortunately there are several candidate vaccines that are in the pipeline. For instance, Feng-Cai and colleagues have reported 90% vaccine efficacy against EV71-associated HFMD and 80.4% efficacy against EV71 associated disease in the phase three clinical trials of their vaccine candidate [14]. While these advances are a step forward in the fight against HFMD caused by EV71, this strategy would not be applicable to all HFMD cases. This is because, in addition to EV71, HFMD has been shown to be widely

associated with *coxsackie A16 virus* (CA16). In a clinical survey conducted by Fan Yang's group across China in 2009, 50.4% of the 266 HFMD cases were identified as EV71 associated cases and 38.3% were CA16 cases [15]. Thus a lot of work is still required on the disease management front. Currently, there is no approved treatment regimen or drug for HFMD [16]. Trials by different scientific groups have been conducted in the past to test the antiviral properties of many compounds like Pleconaril, Ribavarin, Rupintrivir etc [17,18]. However, none of them were effective enough to warrant licensure and were subsequently terminated. As a result of this, disease management has been primarily dependent on patient care and temporary alleviation of symptoms. Thus, the lack of a specific antiviral drug for treatment of HFMD continues to be a major handicap for clinicians and public health workers globally and underscores the dire need for developing effective drugs rapidly.

Traditionally, drug discovery has been a labor, time and cost intensive endeavor and in many cases fails to create an immediate and much needed impact on an infection. Thus, new methods of drug discovery have been developed in recent times and one such approach is drug repositioning [19]. This involves testing of well established drugs as possible candidates for new infections. One such class of drugs is the protease inhibitors. HIV-1 protease inhibitors have been in wide use for treatment of HIV-1 infected patients. Currently there are nine FDA approved HIV-1 protease inhibitors available namely Saquinavir, Ritonavir, Lopinavir, Indinavir, Nelfinavir, Atazanavir, Amprenavir, Darunavir & Tipranavir which are used in various HAART regimens [20]. These drugs are potent inhibitors of the HIV-1 protease enzyme that is essential for processing the viral polyprotein and maturation of viral particles [21]. Specifically targeting this enzyme

hinders the cleavage of the polyprotein which ultimately leads to the formation of non-infectious virions. Through various clinical trials, population and cohort studies, these drugs have been shown to be effective in reducing the viral load and increasing the CD4 T cell counts of HIV-1 infected patients. Hence they are effectively able to slow down the progression of the infection to the AIDS phase [22]. Also numerous biosafety and toxicity studies were conducted to validate drug safety and tolerance. These data thus provide ample evidence of their antiviral capacity and establish them as safe, validated and well documented drug candidates for repositioning.

Additionally, in 2009 Toma, S and colleagues, reported the inhibitory activity of Nelfinavir on the intracellular replication of Hepatitis C virus. They noted that the inhibitory effect was seen at low doses and that the drug failed to cause cytotoxicity and apoptosis [23]. In 2003, Chen, XP and co-workers, observed the lack of Severe Acute Respiratory Syndrome (SARS) in 19 AIDS patients hospitalized during the SARS epidemic [24]. Similar peculiar findings were also reported in the same year by Chan, KS's group in Hong Kong where SARS patients treated with HIV-1 protease inhibitors showed better clinical outcome than those who were not [25]. Based on these observations, Chen, XP's group proposed the possible use of HAART for the treatment and prevention of SARS [26]. Thus, these studies provide additional evidence that HIV-1 PIs can have potential broad spectrum anti-viral effects.

In light of the above mentioned findings, we investigated the possible use of HIV-1 protease inhibitors as antiviral treatment of EV71 infection in vitro.

BACKGROUND

1. Enterovirus 71

1.1 Molecular Epidemiology

As the name suggests, EV71 belongs to the genus *enterovirus* of the *picornaviridae* family of viruses which has human rhinoviruses, coxsackie viruses, poliovirus and hepatitis A virus as prominent members. Phylogenetic analysis of the EV71 revealed 15% divergence within the group and was used to classify EV71 into three distinct groups, A, B and C (Figure.1). After the 2 prominent EV71 epidemics in Bulgaria and Hungary, most of the epidemics were reported from the Asia Pacific region that included Australia and Japan. It was noted that most of the epidemics in these regions were caused by the group B and C strains of EV71. With improved surveillance in eastern countries, it was found that while group B viruses of EV71 were dominant in Singapore and Malaysia, the group C viruses were mostly circulating in mainland China and Vietnam [8]. Molecular typing of the isolates led to further classification of the viruses within the groups.

1.2 Structure and genome

EV71 is a non-enveloped virus containing a 7.4 Kb positive sense, single stranded RNA genome in a capsid composed of four structural proteins, VP1, VP2, VP3 and VP4. There are 60 identical subunits of each protein arranged such that they form a regular icosahedron. Thus, the capsid has five fold axes where 5 VP1 subunits interact, three fold axes where VP1-3 subunits interact and two fold axes where any two of three proteins interact [8,27]. A characteristic picornavirus feature exhibited by EV71 is its

triangulation number (T). Contrary to the theoretically probable particle triangulation number (T)=1, EV71 has a pseudo T=3 arrangement. This is because despite having similar conformations, geometrically these proteins appear to be separate repeating subunits present in a T=3 arrangement. Hence they are said to have a pseudo T= 3 arrangement [27]. Additionally, VP4 is present on the inside tethering the genome to the capsid [8] (Figure.2). Various studies have suggested that the capsid has depressions or canyons near its fivefold vertex and these may be involved in attachment to receptors on host cells [28,29]. To date five host cell receptors associated with EV71 binding have been identified: (i) the scavenger receptor B (SCARB2) which is expressed ubiquitously [30], (ii) human P-selectin glycoprotein ligand-1 (PSGL-1) that is expressed mainly on white blood cells [31], (iii) sialyated glycans found in the gastrointestinal and respiratory tracts [32], (iv) annexin II expressed on human nasopharyngeal mucosa, fetal human brain cells [33] and more recently (v) heparin sulfate that is expressed by all cell types [34]. This widespread expression of most of these receptors may account for the systemic nature of EV71 infection.

1.3 Viral Replication and protein synthesis

Once the viral particle is attached to a host through the binding receptors, the mature virus undergoes a conformational change that transforms it into an intermediate form exhibiting altered antigenic characteristics [28]. This triggers the release of the RNA genome into the host cell cytoplasm via the two fold axis of the icosahedron capsid. It should be noted here that the viral RNA genome is polyadenylated at the 3' end and has a small protein, Vpg linked to its uncapped 5' end. Following its entry into the cytoplasm, the RNA genome is readily translated into a single polyprotein with the aid of the internal

ribosome entry site (IRES) that is located in the 5' UTR of the RNA. In addition to the IRES that enables the RNA translation to occur in a cap-independent manner, host cell factors like IRES-specific transacting factors (ITAFs) are recruited to translate the E71 open reading frame [35]. The polyprotein thus formed undergoes cleavage by viral protease 3C and 2A to yield the 11 structural and non structural proteins which in turn facilitate viral replication. 3D, the viral RNA dependent RNA polymerase, is used to read the RNA genome and replication occurs in a vesicle membrane structure. The structural proteins assemble to form the procapsid and packaging of the RNA genome into the empty capsid results in the formation of an infectious mature virion which then lyse the cell to exit [8] (Figure.3).

1.4 Pathogenesis

The primary route of transmission of EV71 is through the oro-fecal route. However, in a lot of cases, infection can be caused by vesicular fluids, respiratory droplets and oral secretions [9]. Once ingested, typically, virus colonization and replication is restricted to the Peyer's patches, tonsils and to a lesser extent in the local lymph nodes and is thus indicative of the asymptomatic phase of the infection. Overcoming this restraint results in dissemination of the virus to other organs such as the liver, heart, lungs, spleen and the central nervous system, thus marking the start of the symptomatic phase [8].

However, at the molecular level, not much is known about the pathogenesis of EV71 infection. Data from the in vitro studies conducted by Lei and colleagues reported a significant decrease in IFN production by EV71 infected HeLa and RD cells [37]. They showed that the 3C protease, through its association with RIG-I and IPS-1, disrupted the nuclear translocation and subsequent activation of the IRF3 and ultimately down

regulated the production of type 1 IFN in the host cell. In 2011, Lei et al further proposed impairment of TRIF adaptor protein as a second mechanism for 3C protease mediated suppression of IFN production [36]. Interestingly, in their recently published study, Chuanzhen Chi and colleagues observed high levels of IFN- β induction using human colorectal adenocarcinoma cells (HT-29) [37]. Additionally, they also recorded a 45-fold increase in the expression of IL-6 and significant increase in secretion of chemokines like IP10 and CCL5. Cell death through apoptosis is also another mechanism employed by the host immune system to control the spread of infection. Chuanzhen Chi's group found not only an upregulated expression of FasL which can further activate caspase 6 and 7 downstream, but also the activation of caspase 9, indicative of mitochondrial-mediated apoptosis of EV71 infected epithelial cells [37,38]. While these and the aforementioned data are proof of the existence of immune components that are in place to control infection, in a few cases, they are easily overcome by the virus and result in the development of a systemic infection with severe sequelae.

1.4.1 Damage to central nervous system

As in the case of other enterovirus infections, severe EV71 infection patients present with aseptic meningitis, flaccid paralysis and encephalitis, demonstrating the extreme damage it causes to the central nervous system [8]. Several mouse studies and post mortem examinations of fatal human cases implicate retrograde axonal spread of the virus through the peripheral and cranial nerves [39]. Histological examination of tissue biopsies highlights the presence of parenchymal inflammation and perivascular cuffing [8]. Post mortem MRI of EV71 infected children with brainstem encephalitis showed widespread inflammation of the grey matter in the spinal cord and major parts of the

medulla oblongata. Additionally studies have also illustrated the association between EV71 infection, neurological sequelae and cognitive dysfunction [8]. In their study, Chang LN and colleagues found that patients with EV71 infection were more prone to neurological sequelae due to viral invasion and damage of neurons. They also observed that EV71 infected children displayed impaired cognitive functions [40]. However, a direct causal relationship between the two is yet to be determined.

1.4.2 Severe pulmonary edema & Cardiac failure

EV71 has also been shown to be associated with fulminant pulmonary edema. However, whether this is the cause or effect of CNS inflammation remains unclear [8]. Some researchers have suggested that the elevated levels of cytokines such as IL-1 β , IL-6, IL-10, IFN- γ and TNF- α noticed in EV71 infection may contribute to the increased vascular permeability, which in turn causes pulmonary edema. Apart from this, studies have reported pediatric cases with weakened cardiac function [41]. While the data is not sufficient due to the lack of appropriate animal models to study the pathogenesis of the virus, it does highlight the possible connections between EV71 infection and the chronic sequelae observed in patients.

1.5 Vaccines

The successes of the poliovirus and hepatitis A vaccine have ironically been difficult to replicate for EV71. This could be attributed to the non availability of a good animal model to study EV71 pathogenesis. Another concern is the insufficient generation of neutralizing antibody against all three genotypes of the virus. However, interestingly, once infected with a strain, patients develop natural immunity and are protected from further exposures. Many approaches have been adopted to design an effective vaccine:

- a) *Inactivated vaccine*: This conventional vaccine uses generation of neutralizing antibodies as the modus operandi for preventing the infection. Studies by Wu et al showed the antibodies, stimulated by heat killed and formalin-inactivated vaccine, were able to neutralize EV71 infection in neonatal mice on passive transfer. A major drawback for this approach is the dependence of vaccine immunogenicity on the preservation of the viral three dimensional structure. Inactivation reduces the magnitude of the response which in turn can only be restored by increasing the dose or providing adjuvant supplementation. These steps bring in new challenges in terms of stability and vaccine side effects [42].
- b) *Attenuated strain vaccine*: Through their primate studies, Minerato Arita and associates showed the development of broad spectrum neutralizing antibodies on immunization with a 3D polymerase mutated EV71 attenuated strain. While the attenuated strain was not fatal to the monkeys, mild neurological symptoms were observed in the vaccine inoculated animals. Further attenuation should ideally reduce these side effects, but can also potentially cause genetic instability [43]. Another concern with this approach is the possible reversion to the wild type virulent strain.
- c) *Subunit vaccine*: This approach tries to circumvent the challenges of reversion to wild type by using only a subunit of the viral capsid as the immunogen. Wu and colleagues in 2000 provided evidence of successful generation of neutralizing antibody response, CD4 T cell proliferation along with significant induction of IFN γ and IL-10 in mice immunized with a recombinant VP1 protein. EV71 typically colonizes the intestine and making it necessary for the vaccine to be

administered orally to generate mucosal immunity. This raises concerns about the subunit vaccine's capacity to survive this route of administration given that it no longer has the structural integrity of the entire capsid [42]. Also, immunogen purification at a large scale is not cost effective.

- d) *DNA vaccine*: As the name suggests, this approach involves intramuscular administration of DNA segments which when expressed through the host cell machinery produce antigenic proteins that stimulate an immune response. Several groups have investigated the success of this strategy, with Wong Siew Tung's group and Wen-Hao Wu's group being prominent ones. Vaccines from both the groups elicited persistent neutralizing antibodies in mice [44,42]. Additionally, this vaccine type is stable, can be produced with simple DNA isolation methods and is cost effective. However, the presence of limited epitopes in the vaccine often causes the response to be weak and requires boosting. .
- e) *Epitope peptide vaccine*: Here, peptides that have been designed to specifically contain immunogenic epitopes are administered to induce an immune response. The selection of peptides also allows for omission of potential side effects. Damian Guang Wei Foo and colleagues identified 3 specific amino acid sequences in the VP1 protein which elicited T helper cell proliferation along with IL-2 and IFN γ production in the immunized mice. They further showed that this technique could be used to identify B-cell epitopes [45]. Although there are limited data on this front, this approach addresses most of the concerns pertaining to vaccine development.

f) Virus like particle vaccine: This involves generation of immune responses using virus particles that are devoid of their RNA genome but contain all the structural proteins and components as the wild type virus. Yu-Chen Hu and others demonstrated the spontaneous development of the viral like particles from the P1 protein and 3CD expression. These particles were shown to be capable of inducing Th1 as well as Th2 type immune responses [46]. However, the logistics and scale up of these particles need to be determined. [47].

Recently the phase three clinical trial of the inactivated alum adjuvant EV71 vaccine was concluded in China. In the trial carried out by Feng-Cai Z and collaborators, study participants were given an intramuscular dose of the vaccine on day 0 and then on day 28. Vaccine efficacy was 90% against EV71-associated HFMD and 80.4% efficacy against EV71-associated disease. While serious adverse events were seen only in 1.2% of the vaccine administered arm, 71.2% study participants complained of adverse events. Although the results are encouraging, the trial failed to address a few crucial points like cross reactivity. Also, in light of recent data from a sentinel Shenzhen hospital that suggested 80% of the HFMD patients to be children below the age of 5 years [48], the lack of vaccine efficacy data in this demographic is conspicuous [14].

1.7 Treatment

Despite the tremendous progress in vaccine development and focus on prevention strategies, treatment of EV71 infected cases is still crucial for disease control. Many research groups have carried out drug discovery studies with a myriad of compounds all in a bid to find an effective antiviral against EV71 infection. With the resolution of the

virus structure and its life cycle, different stages of the pathogen life cycle are being targeted for inhibition. (Figure.4)

- a) *Drugs targeting viral attachment and entry*: The attachment to host cell surface receptors is pivotal for viral invasion and subsequent replication. Two functionally important receptors namely SCARB2 receptor and PSGL-1 are specifically used by VP1 on the EV71 capsid to attach and invade monocytes, dendritic cells, epithelial cells etc. In addition to these, secondary receptors such as heparan sulfate, sialylated glycans and annexin II are used by the virus to concentrate on the host surface and improve infectivity. Inhibiting this step would impede invasion and subsequently disrupt viral replication. Several therapeutic compounds have been identified and tested for their antiviral efficacy. Both highly *sulfated suramin* and *kappa carrageenan*, a sulfated polysaccharide derived from seaweed specifically target the VP1 protein in the capsid. It is hypothesized that these compounds are able to disturb the structural integrity or steric hindrance of the capsid and thus block binding [34,49]. Seiya Yamayoshi and co-workers displayed binding inhibition in a dose dependent manner using monoclonal antibodies against SCARB2 receptors [30].
- b) *Drugs targeting viral uncoating*: The successful binding of VP1 to the receptors and low pH trigger a series of events in which the capsid undergoes a conformational change to yield an A-capsid that is ready to release its genome into the host cytoplasm. Inhibition of this conformational change would prevent release of the viral genome into the host cells and stall infection. Compounds like BPROZ-194 and pleconaril are pocket binders that have been designed to

specifically bind to the hydrophobic pocket under the canyon depression. However both compounds were found to be ineffective after initial experiments. On the one hand, the occurrence of a single point mutation in VP1 enabled the virus to overcome the binding specificity of BPROZ-194 [50]; on the other hand, pleconaril failed to inhibit the development of cytopathic effect by the 1988 Taiwan EV71 isolate [51].

- c) *Drugs targeting viral RNA translation*: EV71 employs cap independent translation to initiate protein synthesis. IRES in the viral RNA along with ITAFs facilitate translation of the positive sense viral RNA into a single large polyprotein. Zhiqiang Wu and colleagues in 2009 identified several small interfering RNA (siRNA) against the circulating strains in China [52]. While these inhibitors are extremely specific for the amino acid sequence of VP1, they are unable to persist in the plasma due to their short half life. Other inhibitory nucleotides like phosphodiamidate morpho oligomers (PPMO) inhibit the IRES and prevent it from engaging ITAFs for translation. While this strategy recorded a 6 log₁₀ reduction in *poliovirus 1* and *human rhinovirus* titers in infected cell cultures, it is yet to undergo evaluation in the case of EV71 [53].
- d) *Drugs targeting viral polyprotein processing*: Even though translation results in the formation of the polyprotein, functional proteins, required for replication and virion assembly, are only available after proteolytic cleavage of the polyprotein. This is carried out by two viral proteases, 3C and 2A at specific sites in the polyprotein [8]. Additionally, these enzymes have been shown to be important for down regulating type 1 IFN production [37]. Thus, inhibition of these critical

enzymes can be an effective alternative to the current antiviral therapies. Nisrine Falah's group showed that this is possible by using the peptide LVLQTM to block the active site on 2A protease [54]. Another compound that was extensively studied for its inhibitory properties was Rupintrivir, a 3C protease inhibitor originally designed for human rhinoviruses [55]. However, the drug's P1' group could not access the S1 pocket of EV71's 3C protease and thus was rendered ineffective against infection [51]. Other inhibitors that have been studied as 3C protease inhibitors include *fisetin* and *rutin*. These compounds have been shown to possess inhibitory activity at 85 μ M and 100 μ M respectively [56].

- e) *Drugs targeting viral RNA dependent RNA polymerase (RdRp)*: In order to produce infectious progeny, the viral RNA must be used as a template for synthesis of a negative strand which then would facilitate the formation of 2 positive sense strands of RNA. This process is dependent on the VPg protein that must be uridylated to VPg-pUpU which serves as the RNA primer. This is another opportunity to target a crucial stage in the viral life cycle. In 2004, Shih et al identified DTrip-22, a compound that restrained the accumulation of positive and negative sense RNA in the cell. However, a single amino acid substitution in the 3D protein conferred resistance to the drug [57].

Despite the different strategies and targets available, developing an EV71 specific drug has been largely unproductive. Most of the drug candidates, although promising during the in vitro phase, have been unsuccessful in replicating their results during scale up and subsequent clinical trial phases. This is further compounded by the fact that EV71 has an error prone RNA dependent RNA polymerase that has a high mutation rate. While

combination therapy could be a promising tactic to counter such virus mutation strategies, they need extensive evaluation. These challenges when coupled with the sheer magnitude of an epidemic with severe pediatric complications, present a dismal picture of healthcare and EV71 therapy worldwide. Furthermore, they gravely underscore the pressing need for development of new antiviral drugs specific for EV71.

Given the biology and the mutation capacity of the virus, targeting a viral protein that is conserved through all the genotypes and one which is indispensable for virus survival, would theoretically be an ideal approach for developing antiviral drugs. In the case of EV71, non-structural proteins like the 3C and 2A proteases are more likely to be conserved as they are essential for most of the replication processes which include processing of the viral polyprotein, cleavage of precursor proteins to yield other non structural proteins like the 3D polymerase [21]. Additionally, their proteolytic activity is essential for processing polyprotein and forming infectious virions. Thus, targeting these enzymes is an attractive alternative to inhibit viral activity.

2. Protease inhibitors

Protease inhibitors have been successfully employed to target several known viral pathogens. At present, this approach is being extensively used to retard HIV-1 replication. Drugs that specifically target the HIV-1 protease are routinely administered in combination with nucleoside and non-nucleoside inhibitors as a part of the highly active antiretroviral therapy (HAART) to thousands of HIV-1 patients worldwide. This class of drugs has been shown to be effective in significantly reducing the viral load and improving the CD4 count of these patients.

2.1 HIV-1 protease inhibitors

HIV-1 protease inhibitors are peptidomimetic compounds that contain a nonhydrolyzable hydroxymethylene core in place of the traditional peptide linkage. Being small in size, PIs snugly sit in the cleft of the HIV-1 protease homodimer and thus block the active site. This prevents the enzyme from binding to the HIV-1 polyprotein and thus averts cleavage. Rabi SA and colleagues in their recent work on HIV-1 protease inhibitors demonstrated that while the HIV-1 protease inhibitors are unable to prevent the budding out of immature virions, they are able to affect the subsequent virion entry, reverse transcription and post transcriptional steps [22].

Ample evidence of their efficacy and safety has been gathered over the years through population studies and large scale clinical trials. Additionally, being well tolerated with minimum side effects, HIV-1 protease inhibitors have dramatically changed the landscape of HIV-1 treatment. Currently, there are ten FDA approved protease inhibitors namely, Amprenavir, Atazanavir, Darunavir, Fosamprenavir, Indinavir, Lopinavir, Nelfinavir, Ritonavir, Saquinavir and Tipranavir [20]. Owing to remarkable success with viral inhibition, HIV-1 protease inhibitors continue to be the focus of various studies to understand the mechanism involved and their possible application as therapy for other viruses. (Figure.5)

2.1.1 HIV-1 protease inhibitors broad spectrum antiviral activity

One of the initial studies that hinted at the broad spectrum antiviral activity of HIV-1 PIs was by Chan KS and coworkers in 2003. They found that on administering lopinavir/ritonavir along with the standard hospital treatment to 75 severe acute respiratory syndrome-coronavirus (SARS-CoV) patients, there was an overall significant

reduction in the death rate by 2.3% [25] as compared to the controls ($p < 0.05$). Furthermore, in the same year of 2003, during the SARS epidemic in Guangzhou, Xiao Ping Chen and colleagues reported the distinctive absence of SARS-CoV infection in 19 HIV-1 infected patients who were being treated with HAART in the same hospital [24]. These two studies provided preliminary evidence of HIV-1 protease inhibitors being potential antiviral compounds for SARS-CoV.

Later, in 2004, Yamamoto and colleagues screened various compounds to identify potential anti-SARS drugs. Using the FFM-1 CoV strain of SARS isolate from a patient and vero cells, they not only saw inhibition of cytopathic effects in virus infected vero cells, but also an inhibition SARS-CoV virion production post treatment [58].

In 2009, Satoshi Toma's team investigated the effect of nelfinavir on Hepatitis C replication. They noted that on infecting the human hepatoma cell line, Huh7 with a subgenomic replicon plasmid from HCV-N, an infectious clone of genotype 1b, and subsequently treating with nelfinavir resulted in a dose dependent inhibition of intracellular viral replication [23].

More recently, in 2011, Maurizio Federico reported PI-induced inhibition of vesicular stomatitis virus and influenza virus replication. It was observed that the PIs saquinavir and nelfinavir were able to reduce the viral yields by 4 logs. Furthermore, through the experiments, a reduction of 3 logs of influenza virus yield from MDCK-2 cells was noted when treated with HIV-1PIs [59]. These data are suggestive of the broader therapeutic applications of PIs and the need to explore their potency against other viruses. Based on the aforementioned findings and the rationale of targeting conserved proteins of EV71,

this thesis was undertaken to investigate the potential protective efficacy of HIV-1 protease inhibitors against EV71 infection in vitro.

AIM & OBJECTIVES

Aim:

To evaluate the protective efficacy of HIV-1 protease inhibitors against *Enterovirus 71* infection in vitro

Objectives:

- i. Screen different HIV-1 protease inhibitors for antiviral activity against EV71 infection
- ii. Analyze candidate drug for cytotoxicity and effective drug concentration against EV71 infection
- iii. Compare candidate drug's antiviral efficacy against other EV71 strains

MATERIALS & METHODS

1. Cell culture

1.1 Vero cells

African green monkey kidney or vero cells, previously procured from American Type Culture Collection (ATCC), were stored at -150°C in liquid nitrogen. Standardized laboratory protocols for culturing from frozen stocks were followed a few days prior to experimentation. Cells were retrieved from cold storage, thawed in a water bath at 37°C , immediately washed with growth medium to minimize cell death and seeded into a 75cm² T-flask. Dulbecco's modified essential medium (DMEM) supplemented with 2mM L-glutamine, 10% fetal bovine serum (FBS) and treated with 1% penicillin and streptomycin was used as the growth medium. Cells with appropriate volume of growth medium were maintained at 37°C and 5% CO₂ saturation.

For splitting and seeding, vero cells were washed with 1X phosphate buffer saline (PBS) twice and then treated with 1X 0.5% trypsin-EDTA. Excess trypsin-EDTA was discarded followed by incubation of culture flask at 37°C and 5% CO₂ saturation for 5 minutes. Cells were washed with 1ml DMEM supplemented with 10% FBS and seeded as per requirement.

1.2 HB2 cells

HB2 cells or human breast epithelial cells immortalized by the introduction of SV40 genes were obtained from the ATCC and stored at -150°C in liquid nitrogen. Standardized laboratory protocols for culturing from frozen stocks were followed a few days prior to experimentation. Cells were retrieved from cold storage, thawed in a water

bath at 37°C, immediately washed with growth medium to minimize cell death and seeded into a 75cm² T-flask. Dulbecco's modified essential medium (DMEM) supplemented with 2mM L-glutamine, 10% fetal bovine serum (FBS) and treated with 1% penicillin and streptomycin was used as the growth medium. Cells with appropriate volume of growth medium were maintained at 37°C and 5% CO₂ saturation. Standardized laboratory protocol was followed for seeding and splitting cells for the experiments.

1.3 U87MG cells

U87MG or human glioblastoma cells were previously procured from ATCC stored at -150°C in liquid nitrogen till use. Standardized laboratory protocols for culturing from frozen stocks were followed a few days prior to experimentation. Cells were retrieved from cold storage, thawed in a water bath at 37°C, immediately washed with growth medium to minimize cell death and seeded into a 75cm² T-flask. Dulbecco's modified essential medium (DMEM) supplemented with 2mM L-glutamine, 10% fetal bovine serum (FBS) and treated with 1% penicillin and streptomycin was used as the growth medium. Cells with appropriate volume of growth medium were maintained at 37°C and 5% CO₂ saturation. Standardized laboratory protocol was followed for seeding and splitting cells for the experiments.

1.4 THP-1 cells

THP-1 cells or human monocyte-like tumor cells, isolated and cultured originally from an acute monocytic leukemia patient, were purchased from ATCC and stored in the cryofacility. Standardized laboratory protocols for culturing from frozen stocks were followed a few days prior to experimentation. Cells were retrieved, thawed in a water

bath maintained at 37°C and immediately washed with growth medium. The cell inoculum was seeded into a 75cm² T-flask and. Roswell Memorial Park Institute medium (RPMI) supplemented with 2mM L-glutamine, 10% fetal bovine serum (FBS) and treated with 1% penicillin and streptomycin was used as the growth medium. Cells were maintained at 37°C and 5% CO₂ saturation and were cultured at regular intervals.

1.5 U937 cells

The U937 cell line procured from the ATCC is an immortalized cell line isolated and established from a diffuse histiocytic lymphoma patient. These cells primarily exhibit monocytic characteristics making them a suitable in vitro model for antitumor studies. The cell inoculum was seeded into a 75cm² T-flask and. RPMI supplemented with 2mM L-glutamine, 10% fetal bovine serum (FBS) and treated with 1% penicillin and streptomycin was used as the growth medium. Cells were maintained at 37°C and 5% CO₂ saturation and were cultured at regular intervals.

1.6 HeLa cells

The HeLa cell line is a human cervical cancer cell line that is widely used for in vitro analysis of viral infections and testing of antitumor agents. They are essentially adherent epithelial cells that were isolated from an adenocarcinoma patient. Standardized laboratory protocols for culturing from frozen stocks were followed a few days prior to experimentation. DMEM supplemented with 2mM L-glutamine, 10% fetal bovine serum (FBS) and treated with 1% penicillin and streptomycin was used as the growth medium. Cells with appropriate volume of growth medium were maintained at 37°C and 5% CO₂ saturation. A standardized laboratory protocol was followed for seeding and splitting cells for the experiments.

1.7 MRC-5 cells

MRC-5 cells are normal human lung fibroblast cells that were derived from a fetus post 14 weeks of gestation and are usually used for antiviral efficacy testing. The cell line was previously purchased and stored as aliquots in the liquid nitrogen facility. Standardized laboratory protocols for culturing from frozen stocks were followed a few days prior to experimentation. DMEM supplemented with 2mM L-glutamine, 10% fetal bovine serum (FBS) and treated with 1% penicillin and streptomycin was used as the growth medium. Cells with appropriate volume of growth medium were maintained at 37°C and 5% CO₂ saturation. Standardized laboratory protocol was followed for seeding and splitting cells for the experiments.

1.8 MDCK-2 cells

Madin Darby Canine Kidney type 2 cells are a line of epithelial cells derived from the distal tubule in the kidney of a dog and are regularly used to test antiviral drugs owing to their susceptibility to influenza A and B viruses. These cells were kindly lent to the lab by Dr Andy Pekosz and were maintained using DMEM supplemented with 2mM L-glutamine, 10% fetal bovine serum (FBS) and treated with 1% penicillin and streptomycin as the growth medium. For splitting and seeding, MDCK-2 cells were washed with 1X phosphate buffer saline (PBS) twice and then washed with 2X 0.25% trypsin-EDTA twice. Excess trypsin-EDTA was discarded followed by incubation of the culture flask at 37°C and 5% CO₂ saturation for 20 minutes. Cells were washed with 1ml DMEM supplemented with 10% FBS to stop dissociation and were then seeded as per requirement.

2. Virus growth

Enterovirus 71 strains H and BrCr were procured from ATCC and stored at -70°C. Prior to the experiments, virus vials were retrieved from the cold storage and allowed to thaw at 37°C. Both the strains of the virus were propagated in vero cells in DMEM supplemented with 2% FBS. Supernatant from cell culture was harvested on development of cytopathic effects (CPE).

3. Drug Titration

The HIV-1 protease inhibitors Amprenavir, Atazanavir, Indinavir, Lopinavir, Nelfinavir and Ritonavir were obtained from the NIH AIDS reagent program. The lyophilized drugs were reconstituted according to the purity percentage of the reagent stated by NIH such that for a compound of 90% purity, 0.9ml of dimethyl sulfoxide (DMSO) was added to achieve the final concentration of 1mg/ml. Similarly, stock concentration of 0.1M was prepared, aliquoted and stored at -70°C. Prior to use, they were retrieved, diluted down further to desired concentrations using DMSO and DMEM supplemented with 10% FBS. Rupintrivir was used as the positive control as it was designed to specifically fit the core region of the human rhinovirus 3C protease and has also been shown to act against EV71 infection. It must be noted here that for the EV71 3C protease, substrate specificity determinants are not just limited to its core region (S1' to S4 pocket). They extend to region beyond and thereby limit rupintrivir's activity. However, given that this has been the only drug to show significant activity, it was used as a control.

4. Plaque assay

Vero cells were seeded in 6 well dishes and incubated overnight to reach 70% confluency. Virus stock was subjected to tenfold dilutions from 10^{-1} to 10^{-6} using DMEM

supplemented with 2% FBS. The different dilutions were added to the designated vero cell monolayer containing wells and incubated at 37°C and 5% CO₂ saturation for 1 hour. Plates were rocked every 15 minutes to facilitate equal coverage of the monolayer. Once the unabsorbed virus was removed and monolayers were washed with 1X PBS, an overlay of 1% agarose and DMEM was poured over the monolayer in each well. The plates were incubated at 37°C and 5% CO₂ saturation. At the end of 4 days, cells were fixed with formalin, stained with 1% crystal violet dye and then washed under running water. Plaques in each well were counted and used to determine the viral titer of the stock in plaque forming units/ml (PFU/ml). A similar analysis was used to determine the multiplicity of infection (MOI) for the virus.

5. TCID₅₀ assay

Vero cells were seeded into a 96 well flat bottom plate with a concentration of 10³ cells/100uL of DMEM supplemented with 10% FBS. Cells were added to the wells and were incubated overnight to form of a monolayer. Virus stock was subjected to tenfold dilutions using DMEM supplemented with 10% FBS from 10⁻¹ to 10⁻⁹ and was added to the monolayers post incubation. The plate was then incubated at 37°C and 5% CO₂ saturation and examined everyday for CPE. The plate was scored after a period of 3-4 days where wells with CPE were scored positive and those without were marked negative. TCID₅₀ was calculated using the Reed Muench method.

6. Cell counting

Vero cells were first trypsinized for 5 minutes at 37°C and 5% CO₂ saturation, and then washed with DMEM supplemented with 10% FBS. The cell mixture was centrifuged at 1000 rpm for 5 minutes to obtain the cell pellet. The supernatant was discarded and the

pellet was resuspended in 1ml of the medium. Cells and 0.1% trypan blue were mixed in a 1:1 ratio and loaded onto a hemocytometer for enumeration. Cells that appeared bright, devoid of the blue stain and with retention of their round morphology were considered live. Cells that appeared blue were considered dead. Cell concentration (cells/ml) was determined using the standard formula.

7. Antiviral Assay

Vero cells were seeded in 12 well plates at a known concentration of 10^4 cells/ml per well and incubated overnight at 37°C and 5% CO₂ saturation to allow monolayer formation. Cells in the designated wells were then infected with the virus at a known concentration of 0.005 MOI and treated with HIV-1 protease inhibitors at a known concentration of 10µM. Plates were incubated at 37°C and 5% CO₂ saturation and observed for CPE development. On the appearance of CPE, the supernatant was aspirated and stored at -20°C till use. The monolayer was washed and trypsinized to harvest the cells and determine cell viability by trypan blue method. All the assays were either performed in duplicates or triplicates.

8. Time of addition assay

Vero cells were seeded in 12 well plates at a known concentration of 10^4 cells/ml per well and incubated overnight at 37°C and 5% CO₂ saturation to allow monolayer formation. Cells in the -2h wells were pre-treated with 10uM of drug for 2 hours before being infected with the virus at a known concentration of 0.005 MOI. Cells in the 0h wells were infected with the virus and treated with the drug simultaneously. Cells in the +2h wells were initially infected with the virus and then treated with the drug after a 2 hour delay. Plates were incubated at 37°C and 5% CO₂ saturation and observed for CPE

development. On the appearance of CPE, the supernatant was aspirated and stored at -20°C till use. The monolayer was washed and trypsinized to harvest the cells and determine cell viability by trypan blue. All assays were performed in duplicate.

9. Western Blot analysis

Supernatant was harvested from the infected cell monolayers for determining the antiviral effect of the candidate drug. 60µL of Laemeli sample buffer was added to 180µL of each supernatant sample and boiled for 10 minutes to denature the proteins. All samples were vortexed thoroughly and 15µL were subjected to electrophoresis using 0.75mm 12% sodium dodecyl sulfate polyacrylamide gel. Samples along with the appropriate protein ladder were run at 130V for 70 minutes and subsequently the resolved proteins were transferred to a polyvinylidene difluoride (PVDF) membrane through a semi-dry transfer process. Blots were blocked with 5% BSA solution and then probed with a 1:1000 diluted mouse monoclonal antibody against EV71 VP1 protein overnight at 4°C. This was followed by washing and labeling with a rabbit derived anti-mouse HRP conjugated secondary antibody. Blots were developed using NBT and BCIP solutions.

10. Density quantitation of blots

Densitometric analysis of the bands obtained in western blots was carried out using ImageJ, a Java based image processing software package developed by the National Institutes of Health. Density of the blank wells was taken as baseline and relative to them the band density obtained in control and test wells was estimated. Band density was measured in optical density (O.D). These values were then converted to percentages representing the VP1 expression measured in the presence and absence of drug treatment.

11. Cytotoxicity assay

Drug cytotoxicity was determined by the MTS ([3-(4, 5-dimethyl- thiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2- (4-sulfophenyl) - 2H-tetrazolium, inner salt]) (Promega, Madison, WI USA) method. Vero cells were seeded in a 96 well plate at a known concentration of 10^4 cells/100uL per well and incubated overnight at 37°C and 5% CO₂ saturation to allow monolayer formation. Different concentrations of the candidate drug were added to the cells and incubated at 37°C and 5% CO₂ saturation for 4 days. After this, 20uL of the MTS solution was added to each well and incubated at 37°C and 5% CO₂ saturation for 3 hours. An automated plate reader was used to record the absorbance at 490nm. CC₅₀ was determined from the standard absorbance versus dose curve generated.

12. Statistical analysis

CC₅₀ was estimated using both linear and quadratic regression analysis, and EC₅₀ was determined using piece-wise cubic polynomial fitting. Both the values were estimated employing Matlab version 2013 (Mathworks, Natick, MA, USA). A t-test was used for comparison of VP1 protein yields between two groups. Also, a one-way analysis of variance (ANOVA) test was used to compare the VP1 protein yields of the three groups in the time of addition assay. For all the tests, a p value = or < 0.05 was considered statistically significant. The two statistical tests were carried out using Microsoft Excel 2007 (Microsoft Corporation, Redmond WA, USA).

RESULTS

Rationale: Antiviral activity specific to EV71 was determined by the absence of EV71's VP1 protein in the supernatant of cells treated with the drug. This signifies the lack of VP1 formation from the viral polyprotein and the subsequent failure of the virus to produce virions. Therefore, while using western blots to detect VP1 protein, the lack of VP1 protein in the blots is indicative of EV71 inhibition. This is further supported by the absence of cytopathic effects among the cells.

1. Standardization of methods

Vero cells were passaged 3 to 4 times prior to use for any experiment. A single T-25cm² flask containing cell culture yielded 3-4 million cells/ml after every passage. Also cell viability was calculated and found to be 100%.

2. Antiviral activity against: EV71 BrCr strain

2.1. Preliminary screening

Five HIV-1 protease inhibitors at 10µM each were tested for their antiviral activity against EV71 infected vero cells at a concentration of 100TCID₅₀. Of the five drugs tested, amprenavir, atazanavir, indinavir, nelfinavir and ritonavir, nelfinavir exhibited the highest viral inhibition of 83.43% which was comparable to that observed in the case of the positive control drug i.e. rupintrivir. Virus inhibition was seen at a concentration of 10µM in both the cases. While ritonavir showed marginal inhibition of EV71 at 57.81%, indinavir and amprenavir were unable to inhibit the virus and recorded high VP1 yields of 88.98% and 76.33%. (Figure.6a & b)

2.2. Dose dependent screening of Nelfinavir

To further explore the antiviral activity of nelfinavir, vero cells infected with EV71 BrCr strain were subjected to drug challenge with tenfold dilutions of nelfinavir from 10 μ M to 0.0001 μ M. Virus inhibition by nelfinavir was compared to rupintrivir at similar dilutions. Although nelfinavir did inhibit virus at 10 μ M ($p=0.0004$), it failed to completely impede the virus at a lower concentration of 1 μ M as 58% of VP1 protein was detected by the immunoblot. However, this does suggest the existence of antiviral activity of the drug at a concentration that is more than 1 μ M and less than 10 μ M. (Figure.7a & b)

2.2.1. Effective concentration of Nelfinavir

In order to more accurately determine the effective concentration of nelfinavir, EV71 infected vero cells were treated with nelfinavir at the following concentrations: 1, 4, 6, 8, 10, 12 and 14 μ M. On analyzing the immunoblot obtained from the samples, it was confirmed that nelfinavir indeed partially inhibited the virus at 1 μ M. Also, almost complete viral inhibition was seen from 4 μ M onwards. On calculating the density of the VP1 protein obtained with 4 μ M treatment, it was observed that nelfinavir was able to block virus production by 98.4% ($p=0.0057$). Furthermore, there was 100% inhibition of the virus when 6 μ M nelfinavir was used ($p=0.0050$). Additionally, on comparing the inhibition levels obtained with 4 μ M to those obtained with 6 μ M, it was noted that there was no significant difference between them ($p=0.3004$). (Figure.8a & b)

3. Antiviral activity against EV71 H strain

3.1. Preliminary screening

The above mentioned findings prompted testing of HIV-1 protease inhibitors against other strains of EV71. The H strain of EV71 was used to infect vero cells at a concentration of 100 TCID₅₀ and cells were subsequently treated with 10 µM of amprenavir, atazanavir, indinavir, nelfinavir and ritonavir, for investigating possible antiviral activity. The VP1 protein detected in cells treated with nelfinavir was the lowest out of the six protease inhibitors. Only 16.57% VP1 was recorded from nelfinavir treatment, indicative of 83.43% viral inhibition. Among the other protease inhibitors, amprenavir displayed minor antiviral activity by blocking 26.1% virus compared to the controls. Comparatively, atazanavir, indinavir and ritonavir showed no viral inhibition as they recorded high yields of VP1 protein in their western blots: 85.8%, 97.35 and 99.3% respectively. (Figure.9a & b)

3.2. Dose dependent screening of Nelfinavir

In order to precisely resolve the antiviral activity of nelfinavir against EV71 H strain, an increasing dose range of nelfinavir was selected and tested with EV71 H strain infected vero cells. Virus infected cells were treated with 1, 4, 6, 8, 10, 12 and 14µM of nelfinavir and supernatants were analyzed for VP1 yields. While nelfinavir was noted to have limited antiviral activity of 86.68% at 1µM (p=0.00001), greater viral inhibition was observed with increasing concentrations of nelfinavir. At a concentration of 4µM, nelfinavir was found to almost completely inhibit the virus as only 0.24% (p=0.0000001) VP1 protein was detected. On increasing the dose by 2µM, complete or 100% inhibition of the virus was achieved as no VP1 protein was detected when compared to the negative

control ($p=0.0000001$). Additionally, no significant difference ($p=0.3104$) was observed between the virus inhibition level obtained at $4\mu\text{M}$ and $6\mu\text{M}$. (Figure.10a & b)

4. Cytotoxicity and effective concentration determination

The MTS assay was used to examine the cytotoxicity experienced by vero cells exposed to increasing concentrations of nelfinavir. In general, a linear dose dependent response was observed with cell death being recorded at higher doses of nelfinavir. Using linear regression, the dose versus absorbance curve estimated the 50% cytotoxic concentration (CC_{50}) of nelfinavir to be $19.97\mu\text{M}$ or $20\mu\text{M}$ approximately. The root mean square error (RMSE) for this curve model was 0.1311 suggesting a good fit of the curve to the data. Furthermore, to obtain a more precise value of CC_{50} , quadratic regression was carried out which revealed a CC_{50} of $15.65\mu\text{M}$. This model displayed better fit of the curve to the data as the RMSE was 0.0666 (Figure.11a).

The 50% effective concentration (EC_{50}) of nelfinavir against EV71 infection was determined by plotting a dose response curve. Piece-wise cubic polynomial fitting was used for this scenario as linear and quadratic polynomial fitting produced poor R^2 and RMSE values. Also, the nature of curves did not seem to capture the expected physical picture. On plotting the values using this model, the EC_{50} for nelfinavir was estimated to be $1.16\mu\text{M}$ with an R denoting good fit of the model. (Figure.11b)

5. Comparing effect of Nelfinavir on EV71 BrCr and H strain

On comparing the virus inhibition levels obtained with same doses of nelfinavir, both EV71 BrCr and H strain showed no significant difference ($p=0.69$).

6. Time of addition assay

The time of addition assay was carried out to investigate the dependence of antiviral activity on time of addition of drug. This experiment would provide an initial idea about the infection phase dependence of the drug and what would be the ideal phase of treatment. Pre treatment was carried out to see if the drug was able to prevent the entry or uncoating of the virus and delayed treatment was carried out to ascertain the drug's ability to interrupt viral life cycle in an infected cell. Three times points of treatment were assessed namely, (i) nelfinavir treatment 2 hours before infection (-2h), (ii) nelfinavir treatment concurrently with infection (0h) and (iii) nelfinavir treatment 2 hours after infection (+2h). Individually at each time point, nelfinavir treatment resulted in almost complete blockade of the virus with 98.88% ($p=0.0008$), 98.91% ($p=0.0008$) and 97.74% ($p=0.0012$) inhibition levels for (-2h), (0h) and (+2h) groups respectively. Carrying out comparative analysis of the VP1 protein detected at the different time points revealed no difference in the degree of inhibition exerted by nelfinavir. Treatment with nelfinavir at three different time points yielded consistent levels of VP1 protein and the difference in all groups was statistically insignificant ($p=0.7736$). (Figure.12a & b)

7. Therapeutic index of nelfinavir:

Therapeutic index (TI) was used as a measure for the drug's therapeutic potency and was determined using the cytotoxicity and effective concentration values of nelfinavir as $TI_{50} = CC_{50}/EC_{50}$. A high TI is indicative of an effective antiviral capacity with minimum cell toxicity. The estimated TI for nelfinavir was determined to be 13.4813 in this study.

8. Antiviral Activity against other viruses

8.1 HAV

The promising results of nelfinavir's antiviral activity obtained against EV71 infection prompted the exploration of the drug's effect on other members of the picornaviridae family. Hepatitis A virus (HAV) was cultured in vero cells and maintained in DMEM supplemented with 10% FBS.

Vero cells infected with 100 TCID₅₀ of HAV were then treated with 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10µM of nelfinavir and cells were analyzed for HAV infection. As HAV antibody was unavailable, antiviral effect of HAV was estimated by the absence of CPE in cell culture. Cell viability was used to as a measure of the drug's anti- HAV effect.

On the whole, nelfinavir was noted to have significant variable antiviral activity against HAV infection at different concentrations. A comparative analysis of the different levels of virus inhibition obtained with different concentrations (1-10µM) of nelfinavir displayed significant variation among the groups, suggesting the inhibition levels would be dependent on the drug dose ($p=0.000000003$). In the absence of treatment, HAV infection greatly reduced cell viability to 23.13% of the uninfected cells. In contrast to this, maximum cell viability was seen in case of cells treated with 4µM nelfinavir, where 132.43% cell viability was observed. Also, the difference between 4uM treated and untreated cells was seen to be significant ($p=0.000009$). Significant cell viability levels were recorded for cells treated with nelfinavir up to 6µM ($p=0.0002$). However, cell viability was lowest at 22.21% when treated with 10µM nelfinavir and cell death was observed in this case. Also, on comparing the cell viability recorded at this concentration to the negative control, no significant difference was seen ($p=0.7516$). Additionally, on

comparing the cell viability levels obtained with nelfinavir treatment ranging from 1 μ M to 6 μ M, only a marginal difference was noted suggesting, that nelfinavir had a similar antiviral effect ($p=0.024$) in this dose range. (Figure.13a & b)

9. Cytotoxicity evaluation with other cell lines

In order to further examine the cytotoxicity of nelfinavir, possible variation in antiviral effect with the cell type and its effects on specific tumors, a small pilot study was carried out on a 2 panels of cell lines. Panel 1 consisted of tumor cell lines- HB2, U87MG, THP1, U937 and HeLa cells and panel 2 was relatively smaller containing only 2 non-cancerous cells lines namely MRC-5 and MDCK-2 cells. Both panels were treated with 1, 2, 4, 8 and 16 μ M of nelfinavir. A standard concentration of 10^3 cells/100 μ L was used across all the cell lines and high cell viability was used as an indicator of low drug cytotoxicity. For the adherent cell type that included HB2, U87MG, MRC5, HeLa and MDCK cell lines, cells were monitored for CPE everyday for a period of 4 days. On day 4, cells were harvested and cell viability was estimated using trypan blue method. For the non-adherent cell type that included THP-1 and U937 cell lines, cell viability was determined daily for 4 days using trypan blue method. (Figure 14a. & b.)

9.1 Panel I

9.1.1 HB-2 cells

HB-2 or immortalized luminal epithelial cells were found to be completely tolerant to all the above mentioned concentrations of nelfinavir except 16 μ M. Relative to the untreated control cells (blank) only 4.54% viable cells were detected and the difference between them was found to be significant ($p=0.0109$). (Figure 14.c)

9.1.2 U87MG cells

U87MG or human primary glioblastoma cells were noted to be unaffected by almost all of the tested nelfinavir concentrations except 16 μ M ($p=0.5579$). Compared to the untreated well, cells in the 16 μ M well exhibited CPE and only 28.57% were viable. However this difference in viability was not found to be statistically significant ($p=0.1734$). (Figure 14.d)

9.1.3 THP-1 cells

THP-1 cells or leukemic human monocytic cells were treated with the above mentioned dose panel of nelfinavir. In contrast to the untreated cells, cells treated with 16 μ M prominently displayed CPE. While this difference was not statistically significant ($p=0.2475$), a stark numerical difference was noted as only 32% of them were viable relative to the untreated cells. (Figure 14.e.1 & e.2)

9.1.4 U937 cells

This immortalized monocytic cell line was treated with progressively increasing concentrations of nelfinavir and studied for its effect on cell survival. While cell viability remained increased or unchanged up to 8 μ M ($p=0.5431$), a sharp fall in live cell count was recorded in the 16 μ M treatment well as only 35.5% of the cells were viable ($p=0.1554$). (Figure 14.f.1 & f.2)

9.1.5 HeLa cells

The human cervical cancer cell line was also treated with same range of nelfinavir concentrations as stated above and was observed for morphological changes and cell death over a period of 4 days. Cells that were treated with a higher dose of nelfinavir i.e. 16 μ M were no longer detectable (0%) under the microscope suggesting they had severely

diminished in numbers and this reduction in cell viability was found to be statistically significant ($p=0.0210$). (Figure 14.g)

9.2 Panel II

9.2.1 MRC-5 cells

The non-cancerous human lung fibroblast cell line was subjected to nelfinavir treatment at different concentrations to assess the cytotoxicity to cells. Cell viability was only 4.54% in the 16 μ M treated cells as opposed to the untreated cells that served as the control ($p=0.0377$). However, there was no significant decrease in the cell viability at the lower concentrations of the drug ($p=0.8492$). (Figure 14.h)

9.2.2 MDCK-2 cells

MDCK-2, the second cell line in the non-cancerous panel, was primarily composed of epithelial cells found in canine kidney. These cells displayed a nelfinavir tolerance capacity up to 8 μ M dosage. However, on treating them with a higher drug concentration of 16 μ M, CPE was noted in addition to a significant reduction in viable cell number ($p=0.0111$). (Figure 14.i)

An overall comparison of both the cell line panels showed 16 μ M to be the least tolerated dose of nelfinavir as all cell types exhibited major reduction in viability relative to their respective untreated controls and no statistically significant variation was found among this subset ($p=0.2920$). (Figure 14.a & b)

This analysis also revealed a very interesting and unusual trend in both the cell panels. In all the cell lines, treatment with nelfinavir at lower concentrations (i.e. up to 6 μ M) resulted in a small boost in the viable cell number to the extent that they were found to be more than their respective untreated control cells. In panel I (i.e. HB-2, U87MG, THP-1,

U937 & HeLa cells), the increase in cell viability percentage was seen throughout and was highly significant in case of HB-2, THP-1 and HeLa cells ($p=0.0042$, 0.0020 , 0.0022 respectively). In panel II, although cell viability of MRC-5 and MDCK-2 cells did quantitatively exceed the viability levels in their corresponding untreated controls, this variation was not statistically significant ($p=0.8492$, 0.2561 respectively).

DISCUSSION

Enterovirus 71, the etiological agent of Hand, foot and mouth disease, is a major public health issue especially for young children in the first five years of life [8]. Most of the recent epidemics have been reported from the eastern hemisphere with a large proportion of them being from China [61]. Outbreaks of the virus were recorded across the country in 2008 with Anhui province being the epicenter with approximately 490,000 cases. More recently in 2012, 2,168,737 confirmed cases of HFMD were documented by the Chinese Center for Disease Control and Prevention in mainland China [61, 62]. The occurrence of the epidemic has also been noted in other countries, such as Thailand, Singapore and Vietnam to name a few [5,6,7]. Although mostly self resolving, the virus can cause complications and lead to chronic sequelae, such as severe encephalitis, pulmonary edema and heart failure [8].

To date there is no licensed vaccine or drug available as an effective intervention strategy with patients being completely dependent on care management for temporary relief from symptoms. Given the scale of the epidemic, the severity of the pathophysiology, and the lack preventive and treatment strategies, research to develop an effective antiviral drug against EV71 infection is deeply warranted. Many drugs, utilizing varying mechanisms, have failed in the past to demonstrate efficacy against EV71 infection due to a myriad of reasons, including the inability to specifically block VP1 protein and mutations in the hydrophobic binding pockets. In this project, we reason that targeting viral enzymes, such as 3C or 2A proteases would be more productive because in addition to being indispensable for virus replication, these viral proteins are more likely to be conserved in all the different subtypes of EV71.

One of the biggest challenges of drug development is identification of an active substance that has a good safety and bioavailability profile. A strategy that has been successfully adopted in the past to treat health problems like rheumatoid arthritis, Cushing syndrome and HIV-1/AIDS is drug repositioning [19]. Repositioning allows for accelerated investigation of existing drugs, which have previously established safety data, against new diseases and health conditions.

Based on the above mentioned rationale and the success of repositioning, we chose to screen various HIV-1 protease inhibitor compounds that have been effectively used in HAART for HIV-1 infected individuals, for activity against EV71 infection.

In this project, we have shown that vero cells infected with EV71 BrCr and H strains can be effectively used as an in vitro model to assess the activity of HIV-1 protease inhibitors. Our preliminary screening of five different HIV-1 protease inhibitors revealed nelfinavir to have potential antiviral activity against EV71 infection in vitro. This initial inspection phase allowed us to identify and focus on a single candidate drug. While no studies to date have examined the action of HIV-1 protease inhibitors on EV71 infection, they have been tested against other RNA genome based viruses. Norio Yamamoto and colleagues in 2004 investigated the applicability of various anti-HIV-1 drugs and found nelfinavir to inhibit SARS associated coronavirus. They showed the drug to have strong inhibitory action against viral replication in addition to being a deterrent to virus induced cytopathic effects [58]. We obtained similar results in our study where nelfinavir was able to impede EV71 infection effectively. These preliminary findings prompted further analysis of the nelfinavir's antiviral activity.

To gain a better understanding of nelfinavir's antiviral activity, tenfold serial dilutions of the drug were uniformly challenged with EV71 BrCr of known concentration and compared to the antiviral activity of the positive control drug, rupintrivir. It must be noted that rupintrivir is a peptidomimetic compound designed to inhibit the 3C protease enzyme in *human rhinovirus* (another enterovirus) and was thus taken as the positive control for this study. A declining antiviral activity was detected, with maximum virus inhibition of 100% recorded with treatment with 10 μ M. It should be noted here that a partial antiviral activity of 58% was detected at 1 μ M treatment. This was indicative of the existence of dose dependent inhibition of EV71 BrCr by nelfinavir. Also when nelfinavir's drug action was compared to rupintrivir on a dose by dose basis, it was observed that there was no significant difference ($p=0.199725$) between the two drugs. This suggested that nelfinavir's antiviral action was comparable to that exhibited by rupintrivir and thus warranted further investigation of its antiviral activity against EV71 infection.

These experiments hinted at nelfinavir being a potential broad spectrum antiviral drug and strengthened our case. As seen with any other drug candidate, the 50% effective concentration or EC₅₀ is essential to express and describe the drug's ability to block the virus by half. Yamamoto et al determined the EC₅₀ of nelfinavir against SARS-coronavirus to be 0.024 μ M [58]. In contrast, in another study that examined the effect of nelfinavir on HCV replication, Toma S et al estimated the EC₅₀ to be 9.88 μ M [23]. Our study revealed the EC₅₀ of nelfinavir to be 1.1610 μ M. In the absence of a clinical trial the therapeutic implications of this finding are still undetermined; however, the EC₅₀ in our study is very close to the plasma concentration range observed during the clinical use

of nelfinavir (i.e. 3.3 to 6.0 μM) against HIV. This is clearly indicative of the fact that the effective dose is well within the tolerance range of humans.

This was further confirmed by the cytotoxicity concentration of nelfinavir required to reduce cell viability by 50%. Our study revealed CC_{50} of nelfinavir to be 15.65 μM . These findings were however not in accordance with the results obtained by Yamamoto and co workers (CC_{50} =2.75 μM) [58]. In another study done to assess the effect of nelfinavir as an inhibitor of Kaposi's sarcoma associated herpes virus replication, nelfinavir was not cytotoxic at concentrations less than 20 μM [63]. The variation seen among the different studies could be attributed to the use of different methods to evaluate cytotoxicity. While we used the MTS assay to study the cytotoxicity, both the Yamamoto group and Gantt group employed the MTT method.

In addition to these factors, the therapeutic index of nelfinavir was determined to be 13.5 and this was used as a measure for its therapeutic potency. However, a higher value of 302.1 therapeutic index or selectivity index was reported by Yamamoto et al when testing against SARS-CoV [58]. In another study that tested nelfinavir's therapeutic application on *vesicular stomatitis virus* and *influenza virus*, therapeutic index values of 22 and 15 were calculated respectively [59]. This difference in values can be ascribed to the variation seen in the EC_{50} and CC_{50} for different viral infections tested and their corresponding effect on the therapeutic index value.

The drug action was not restricted to a single strain of EV71. To ascertain that the antiviral effect was a pan EV71 phenomenon, all the assays were repeated for vero cells infected with EV71 H strain. We observed similar and in some cases higher levels of virus inhibition when treated with nelfinavir. Furthermore, statistical analysis revealed no

difference in the inhibition levels obtained by nelfinavir action of the BrCr strain and H strain of EV71. These data underscore the 'umbrella inhibitory effect' nelfinavir has on EV71 infection in vitro. Other studies done on nelfinavir have only recorded its action against a single study virus and strain, thereby limiting the scope of their findings. With these results our study highlighted the potential broad antiviral property of nelfinavir.

In addition, one of the objectives of our study was to investigate the timing of nelfinavir treatment and its effect on the outcome. It was found that irrespective of when the drug was added to the cells, virus inhibition was seen in all the three groups (i.e. -2h, 0h and +2h). This observation suggests that nelfinavir may not be specifically acting to inhibit entry of the virus per se. Having said that, it also not clear if indeed there is inhibition of viral entry. Experiments specifically detecting the inhibition of viral RNA in the cell would provide better evidence. Also, the fact that inhibition was seen in the delayed treatment group alludes to nelfinavir's possible impediment of the viral life cycle in virus infected cells. Again, as mentioned earlier, whether this can be interpreted as an inhibition of the polyprotein processing steps or replication remains unclear. This initial observation requires further experimental verification to conclusively state nelfinavir's role. However, these data do suggest that drug treatment appears to be independent of the time of treatment or virus exposure.

The promising results obtained with the two different strains of EV71 prompted us to ask the next logical question of whether nelfinavir would have any action against other members of the picornaviridae family. To address this question, vero cells were infected with hepatitis A virus and treated with nelfinavir. Also, to reduce the number of potential variables and confounders affecting the study, the same cell concentration, drug dose

panel and experimental conditions were maintained for the HAV experiment. Emergence of CPE in cells was the experimental end point and was indicative of the therapeutic failure of the drug. As seen in the case of EV71 infection, HAV infection was inhibited in cells treated with nelfinavir. Antiviral effect was seen 2 μ M onwards with the highest being seen at 4 μ M (130%). Cells at these concentrations had no change in their morphology and lacked CPE completely suggesting a possible anti-HAV effect of the drug.

Although these experimental results highlight the positive therapeutic nature of nelfinavir against EV71 and HAV, it is crucial to grasp the importance of this finding in the light of the current status of treatment interventions available. While many may argue that these infections are self limiting and can be managed with supportive care, in a lot of cases and especially those with complications, the lack of therapeutic tools results in delayed recovery, prolonged morbidity and in some cases death. In their study on the neurological manifestations of EV71 infection, Peter McMinn's team reported the poor effect of intravenous immunoglobulin (IVIG) therapy containing neutralizing antibodies to EV71 [64]. Similar findings were seen in by Huang CC and co workers in 34 patients with culture positive EV71 infection [65]. In another study, Luan-Yin Chang and co workers noted that although IVIG was given, it failed to inhibit sequelae, progression to cardiopulmonary failure and had no appreciable benefit when compared to the untreated group [66].

Similarly, type-I IFN therapy had earlier been proposed as therapeutic option owing to its general antiviral properties. However, murine studies later showed type I IFNs to be ineffective when administered after the infection [67]. Furthermore, in the clinical setting,

the protective effect is marginal [68]. Apart from this, steroids have also been tried as therapy for EV71 infections but have been met with little success. In their 2009 EID report, Sophie Vallet and colleagues described the rapid deterioration of a 17 month old boy who was given symptomatic medication and oral rehydration for EV71 infection. Despite, the administration of intravenous corticotherapy along with aerosol and oxygen therapy within 12 hours of discharge, the patient was unable to recover and died [69].

Milrinone, a phosphodiesterase inhibitor that is commonly used in cardiac failure patients has also been tested as a candidate drug for the treatment of EV71 infection and associated pathology. In the recently concluded randomized milrinone therapy clinical trial, Ching-Chuan Liu's group showed that milrinone was able to reduce the EV71 induced pulmonary edema associated mortality. However, a limitation to this approach is the absence of antiviral activity of the drug per se which would greatly restrict its use [70].

While the aforementioned drugs have been tested in human subjects, efficacy testing of ribavirin has been restricted to in vitro studies. Guofeng Zhang's group studied the presence of antiviral activity for ribavirin in RD cells. However, the drug displayed a very high value of EC_{50} (89.23 to 178.42 $\mu\text{g/ml}$) [71]. Although the Zhang group also tested pleconaril, a capsid binding drug, and highlighted its anti-EV71 efficacy, Kak-Shan Shia's study revealed the drug's inability to neutralize CPE induced by the 1998 Taiwan outbreak strain of EV71 [72].

In comparison to all these drugs, we found nelfinavir to be suitable drug option for EV71 infection owing to its low EC_{50} seen in our study. Additionally, it is able to directly affect the virus and cause inhibition, unlike steroids and IVIG. Also, it has been shown to be

effective irrespective of the time of administration i.e. before or after infection. Moreover, our study was also able to provide preliminary clues about the relationship between drug treatment, antiviral activity and time of infection.

Another, viral infection that usually takes months to resolve if untreated is hepatitis A virus. In some cases it may cause fulminant hepatitis and cholestasis. For instance, Nicola Coppola's group in 2007 reported a case of acute HAV with severe cholestasis that was not resolved by antibiotic treatment and a long course of prednisone [73]. In light of these facts and the data generated in our pilot study, we speculate that nelfinavir could be a probable anti-HAV drug. Having said that, ours is a preliminary study and this aspect of the drug requires further investigation.

An intriguing aspect of the drug that was observed in the course of this study was the effect of nelfinavir on tumor cell lines. Traditionally, nelfinavir was designed to inhibit the viral protease enzyme. However, increasing research and the conspicuously low rates of HIV associated cancers among patients on HAART, indicated anticancer implications for HIV protease inhibitors. In their seminal paper, the Gills' group first demonstrated nelfinavir to be an anti-neoplastic agent inducing autophagy, endoplasmic reticulum stress and apoptosis both in vitro and in vivo [74]. Out of the six HIV-1 protease inhibitors (nelfinavir, saquinavir, ritonavir, indinavir and atazanavir) tested on two non-small cell lung cancer cell lines, namely A549 and H157, they found nelfinavir to be the most potent with mean 50% growth inhibition at the 8 μ M and 9 μ M concentrations respectively. Furthermore, they tested 60 other tumor cell lines and reported nelfinavir to have a mean cytotoxic concentration of 5.2 μ M, in addition to being cytotoxic at a concentration of ≤ 10 μ M in 14 cell lines [74]. Subsequently in another study by M Kraus

and colleagues, nelfinavir was shown to be cytotoxic to myeloma cells namely U266, AMO-1, LP-1 and RPMI8226 at the 20 μ M - 40 μ M concentration [75].

Similar findings with respect to nelfinavir cytotoxicity were observed in the present study. Five cancerous human cell lines i.e. HB-2, U87MG, THP-1, U937 and HeLa cells were used to represent a range of cancers namely breast cancer, glioblastoma, acute monocytic leukemia, histiocytic lymphoma and cervical cancer. When these cell lines were tested with increasing concentrations of nelfinavir, it was noted that all of them were inhibited at concentrations at or above 8 μ M. Also, there was drastic drop in the cell viability at a higher concentration of 16 μ M of nelfinavir, suggesting that the 50% growth inhibition concentration would lie in this range. It should be noted here, that these findings are also in accord with another study that evaluated the inhibitory concentration of nelfinavir for glioblastoma and is currently undergoing clinical trial. In comparison to their inhibitory concentration of 20 μ M, the current study gives as a more specific range of the concentration [76]. A comparable trend was seen in the case of MRC5 cells and MDCK cells.

In addition to these findings, an unusual pattern was recorded in this study. While overall the trend of cell viability suggested a progressive decline with increasing concentration of the drug, a more careful analysis of the curve revealed an initial increase or boost in the cell viability. This significant increase in cell viability was more when compared to the cells in the untreated control well and was mostly seen in all cells treated with up to 6 μ M of nelfinavir. Whether this observation could explain the rise in CD4 cell count on initiation of HAART in HIV infected patients remains to be investigated.

To grasp a better understanding of the findings, it should be noted that the C_{\max} for nelfinavir in HIV patients ranges from 7-9 μ M [74] and in this study nelfinavir was found to most potent between 8-16 μ M. This combined with the fact that it is surprisingly not cytotoxic at lower concentrations leads to us to hypothesize that nelfinavir is a potential broad-spectrum drug that has a dose dependent anticancer effect.

CONCLUSION

In summary, despite being one of the most widely occurring epidemics that lacks effective prevention and control tools, EV71 induced hand, foot and mouth disease continues to be the proverbial elephant in the room. Through this in vitro study we have tried to address the compelling question of effective treatment for EV71 infection. We identified nelfinavir, an HIV-1 protease inhibitor as a potential candidate and demonstrated it to have high protective efficacy along with low cytotoxicity. The study highlighted nelfinavir's ability to significantly inhibit two different strains of EV71 thereby eliciting a pan-EV71 antiviral activity. Additionally, it shed light on nelfinavir's extended antiviral applications to HAV infection and its antitumor effects, thereby suggesting it to be a potential broad spectrum drug. The findings of this pilot study provide the basic groundwork for more advanced experiments aimed at finding a potent drug against EV71.

FUTURE DIRECTIONS

There are a few important research questions that remained unanswered at the end of our study and would be considered while designing future experiments. Although it was beyond the scope of this pilot study, the study was unable to expertly provide a mechanistic explanation of nelfinavir's broad spectrum antiviral activity especially in the case of EV71 and if its activity was greater than that shown by rupintrivir. The existence of structural similarity between the HIV and enterovirus proteases which could account for this inhibition or presence of unique protein folding needs to be determined. In order to strengthen the proposed model of EV71 inhibition by nelfinavir, the molecular basis of the inhibition needs to be better understood.

Currently we are in the process of testing nelfinavir's antiviral activity against other member viruses of the picornaviridae family including coxsackie A16. Also as it has been found to be well tolerated by MDCK cells, we are also testing its possible effect on influenza virus in an endeavor to open up treatment options to infectious disease causing viruses with a similar premise. Another crucial aspect of the study that requires further investigation is the pathophysiology of EV71 infection and how late into the infection nelfinavir therapy can be started. Finally, at the pharmacokinetic level, we would like to analyze the possibility of generating nelfinavir derivatives and investigate their efficacy compared to nelfinavir.

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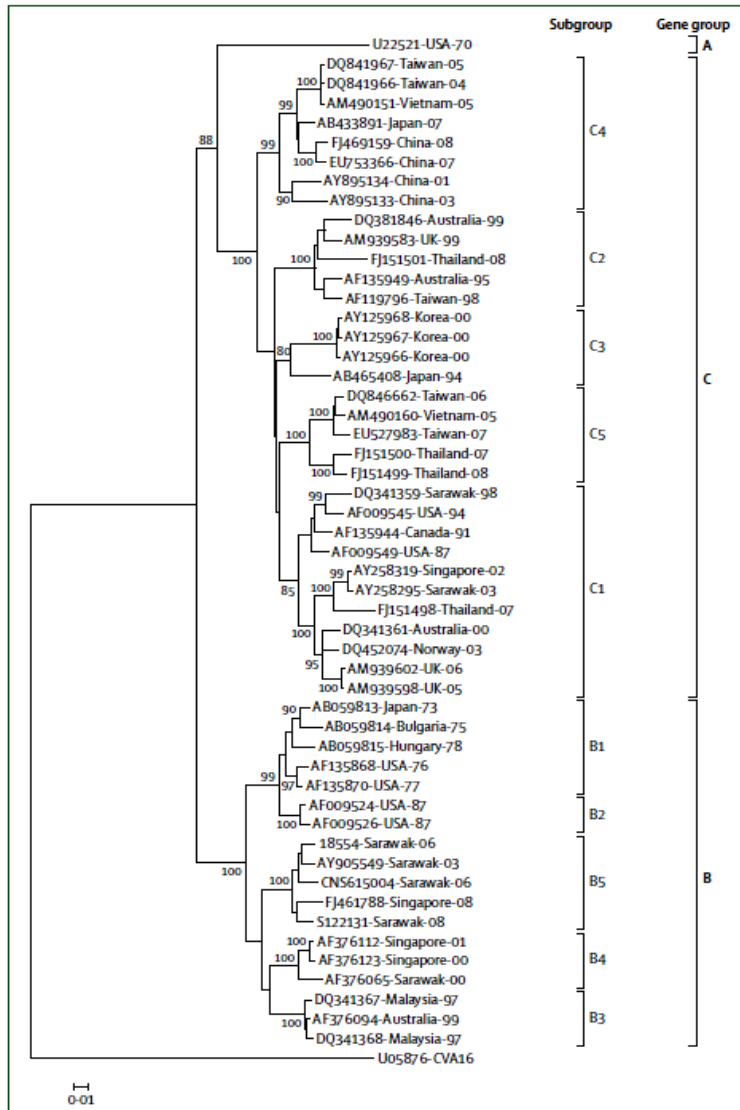
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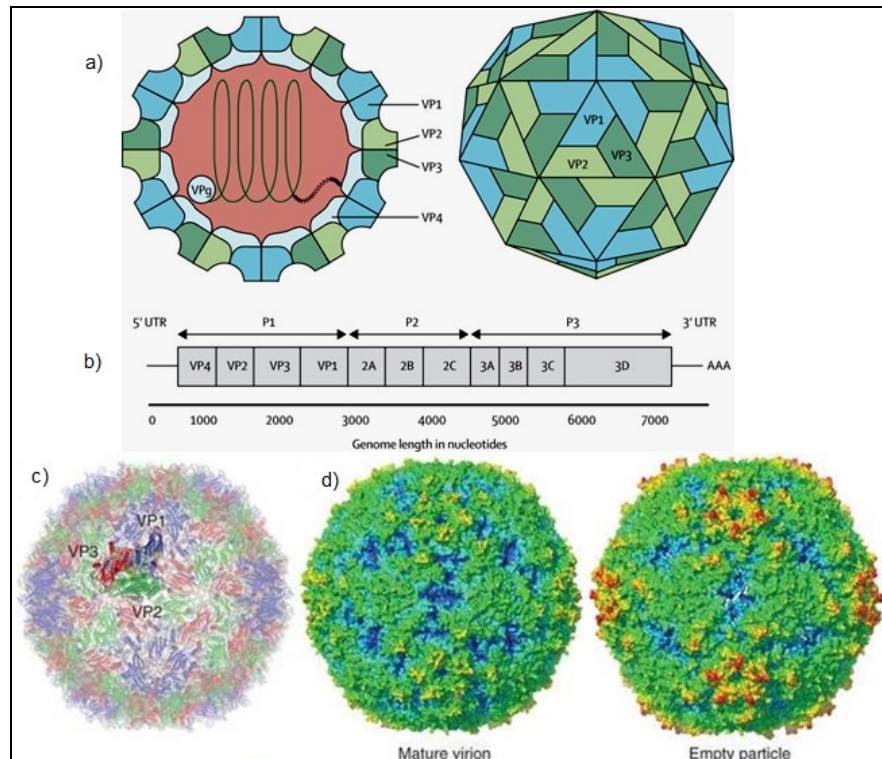
Figure.1 : Phylogenetic analysis of enterovirus 71 VP1 gene sequences

A neighbor-joining tree constructed with the Kimura-2 parameter as a model for nucleotide substitution. The robustness of the tree was determined by bootstrapping, with use of 1000 pseudoreplicates.



Source: Solomon, T., Lewthwaite, P., Perera, D., Cardosa, M. J., McMinn, P., & Ooi, M. H. (2010). Virology, epidemiology, pathogenesis, and control of enterovirus 71. *Lancet Infect Dis*, 10(11), 778-790. doi: 10.1016/S1473-3099(10)70194-8

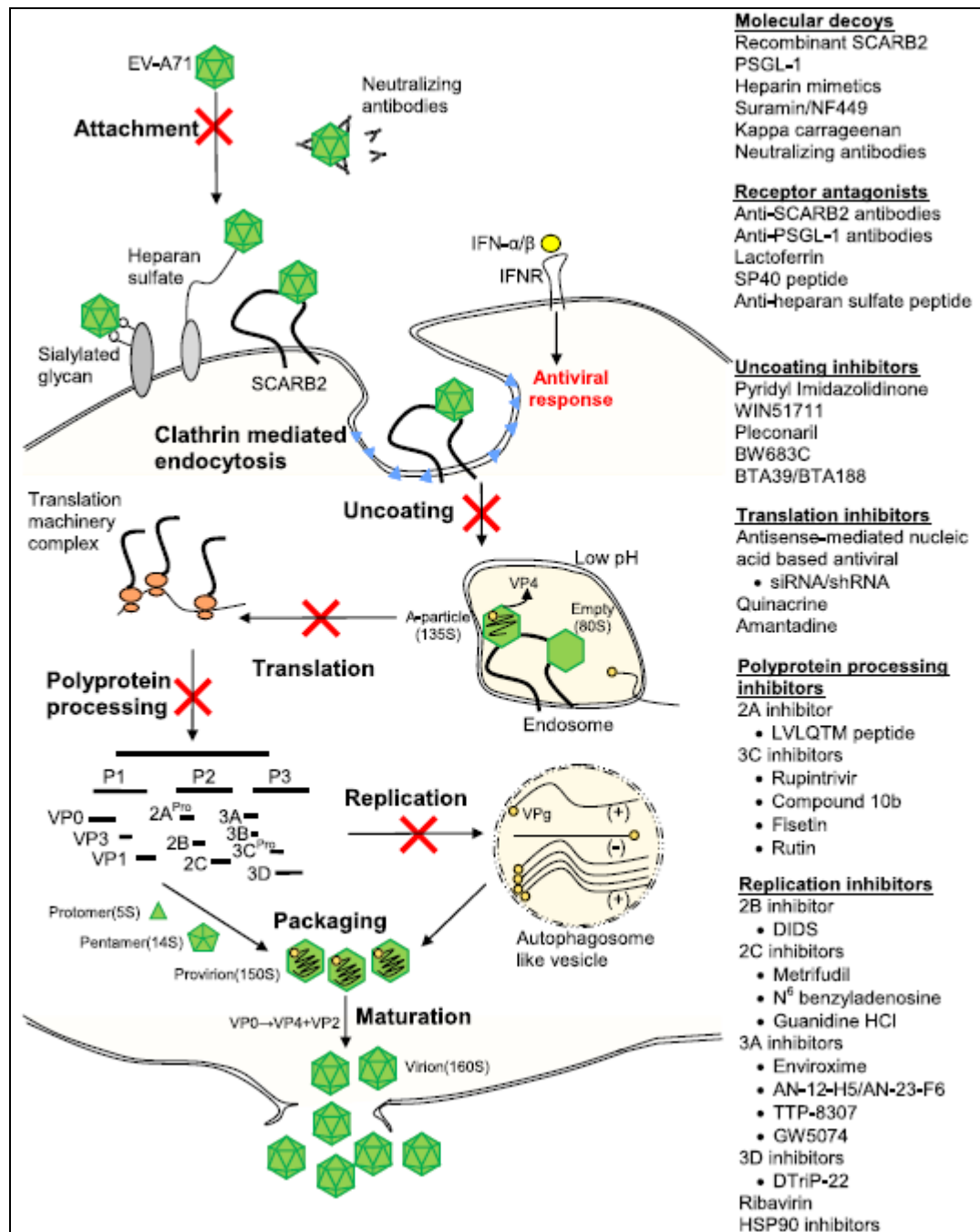
Figure.2 : Enterovirus 71 virion structure and genome layout



Human enterovirus 71. (a) Schematic of virion structure. Each protomer in the virus capsid contains four structural viral proteins (VP1–VP4). (b) Viral genome layout. All the structural proteins are encoded by the P1 region, while P2 and P3 regions encode seven non-structural proteins (2A–2C) and (3A–3D). UTR=untranslated region. VPg=virus encoded protein (c) Ribbon model representation of a single icosahedron unit formed by the interaction of VP 1,2 and 3 in the EV71 capsid (d) Radius-colored surface representation of the EV71 mature particle. The surface of the mature virion is colored to represent the distance from the center; blue denotes subunits closer to the center and red denotes subunits farthest from the center.

Source: Solomon, T., Lewthwaite, P., Perera, D., Cardoso, M. J., McMinn, P., & Ooi, M. H. (2010). Virology, epidemiology, pathogenesis, and control of enterovirus 71. *Lancet Infect Dis*, 10(11), 778-790. doi: 10.1016/S1473-3099(10)70194-8; Wang, X., Peng, W., Ren, J., Hu, Z., Xu, J., Lou, Z., . . . Rao, Z. (2012). A sensor-adaptor mechanism for enterovirus uncoating from structures of EV71. *Nat Struct Mol Biol*, 19(4), 424-429. doi: 10.1038/nsmb.2255

Figure.3 : Schematic illustration of EV71 life cycle and antiviral agents



Source: Tan, C. W., Lai, J. K., Sam, I. C., & Chan, Y. F. (2014). Recent developments in antiviral agents against enterovirus 71 infection. *J Biomed Sci*, 21, 14. doi: 10.1186/1423-0127-21-14

Figure.4: List of antivirals against EV-71 infection tested in vitro & in vivo

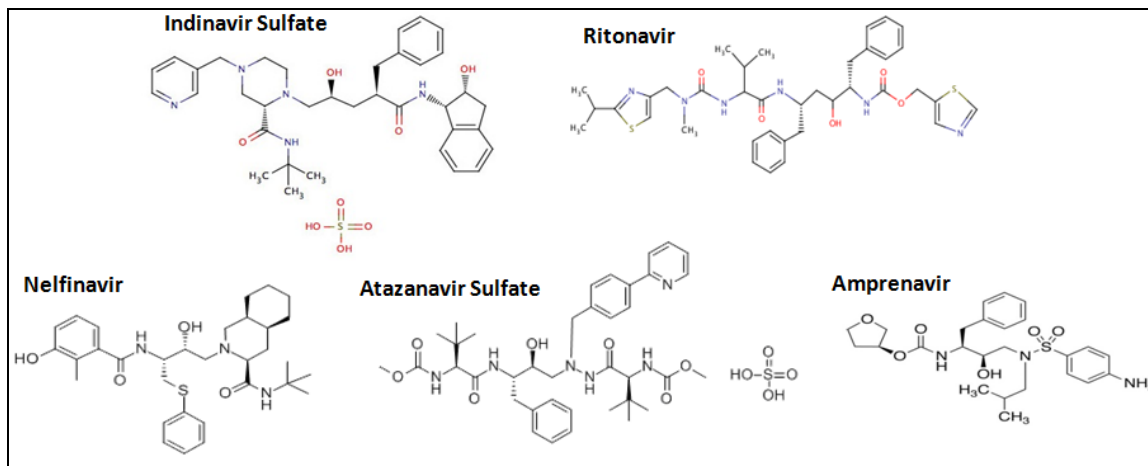
Antivirals	EV-71 genotype tested	IC ₅₀ /EC ₅₀	In vitro cell type	Resistant mutants	In vivo mouse model
Therapeutics targeting viral attachment and entry					
Molecular decoys					
Recombinant SCARB2	B3	N/R	RD		
PSGL-1	C2	N/R	L-PSGL-1.1		
Heparin mimetics					
Heparin	C2	205 µg/ml	Vero, RD		
Heparan sulfate	C2	290 µg/ml	Vero		
Pentosan polysulfate	C2	238 µg/ml	Vero		
Dextran sulfate	B4	N/R	RD		
Suramin/NF449	B1, B3, B4	6.7 µM	RD	VP1 E98Q, K244R	
Kappa carrageenan	B4	N/R	Vero		
Enviroxime-like compounds					
AN-12-H5	B1	0.55 µM	RD	VP1 M119L, VP3 R227K	
AN-23-F6	B1	0.15 µM	RD	VP1 A224T	
Receptor antagonists					
Anti-SCARB2 antibodies	B3	N/R	RD		
Anti-PSGL-1 antibodies	B3, B4, C1, C2, C4	N/R	Jurkat		
Bovine lactoferrin	C2, MP4 ^a	10.5 – 24.5 µg/ml	RD, Vero, SK-N-SH		17-days old ICR
Human lactoferrin	N/R	103.3 – 185.0 µg/ml	RD, Vero		
SP40 peptide	A, B4, C2	6 – 9.3 µM	RD, HeLa, HT-29, Vero		
Anti-heparan sulfate peptide	B4	N/R	RD		
Therapeutics targeting viral uncoating					
Pyridyl imidazolidinone					
BPROZ-299	C2	0.02 µM	RD	VP1 V192M	
BPROZ-284	A, B1, C2	0.04 µM	RD		
BPROZ-194	C2	1.552 µM	RD	VP1 V192M	
BPROZ-160	C2	0.011 µM	RD	VP1 V192M	
BPROZ-112	A, B1, C2	0.04 µM	RD		
BPROZ-103	C2	0.13 µM	RD	VP1 V192M	
BPROZ-101	A, B1, C2	0.0012 µM	RD		
BPROZ-074	A, B1, C2	0.0008 – 0.018 µM	RD	VP1 V192M	
BPROZ-033	A, B1, C2	0.0088 – 0.069 µM	RD		
WIN51711	B3	N/R	RD		
Pleconaril	A	0.13-0.54 µg/ml	RD		1-day old ICR
BW683C	A	> 10 µM	HEp-2		
Compound 3 g	A	0.45 µM	HEp-2		
BTA39	A	0.001 µM	Vero		

Figure.4: List of antivirals against EV-71 infection tested in vitro & in vivo (contd.)

Antivirals	EV-71 genotype tested	IC ₅₀ /EC ₅₀	In vitro cell type	Resistant mutants	In vivo mouse model
BTA188	A	0.082 µM	Vero		
Therapeutics targeting viral translation					
RNA-based therapeutics					
siRNA	B4	< 1 nM	RD		1-day old Balb/c
shRNA	B4	< 1 nM	RD		1-day old Balb/c
Quinacrine	C4	9.71 µM	RD		
Amantadine	Pseudo-EV-71	N/R	COS-1		
Therapeutics targeting viral polyprotein processing					
2A inhibitor					
LVLQTM peptide	C4	9.6 µM	HeLa		
3C inhibitors					
Rupintrivir	C4	0.014 µM	RD		2-days old ICR
Compound 10b	C2	0.018 µM	RD		
Fisetin	CMUH01*	85 µM	RD		
Rutin	CMUH01*	110 µM	RD		
Therapeutics targeting viral replication					
2B inhibitor					
DIDS	C4	N/R	RD		
2C inhibitors					
Metrifudil	B1	1.3 µM	RD	2C E325G	
N ⁶ benzyladenosine	B1	0.1 µM	RD	2C H118Y, I324M	
Guanidine-HCl	B3	N/R	RD	2C M193L	
3A inhibitors					
Enviroxime	A	0.15 µM	Vero		
AN-12-H5	B1	0.55 µM	RD	3A E39G	
AN-23-F6	B1	0.15 µM	RD		
TTP-8307	A	> 60 µM	Vero		
GW5074	B1	6.4 µM	RD		
3D inhibitors					
DTriP-22	A, B2, C2	0.3 µM	RD	3D R163K	
Ribavirin	C2, M2 ^b	266 µM	RD, SK-N-SH, N18	3D G64R, S264L	12-days old ICR
Heat-shock protein 90 inhibitor					
Geldanamycin	B4, C2	N/R	RD		
17-AAG	C2, C4	N/R	N/R		7-days old hSCARF-Tg C57BL/6 mice

Source: Tan, C. W., Lai, J. K., Sam, I. C., & Chan, Y. F. (2014). Recent developments in antiviral agents against enterovirus 71 infection. *J Biomed Sci*, 21, 14. doi: 10.1186/1423-0127-21-14

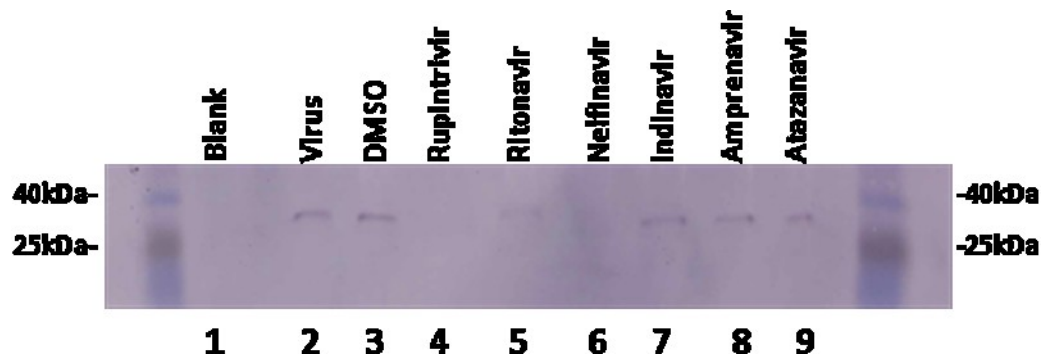
Figure.5: HIV-1 protease inhibitor drugs



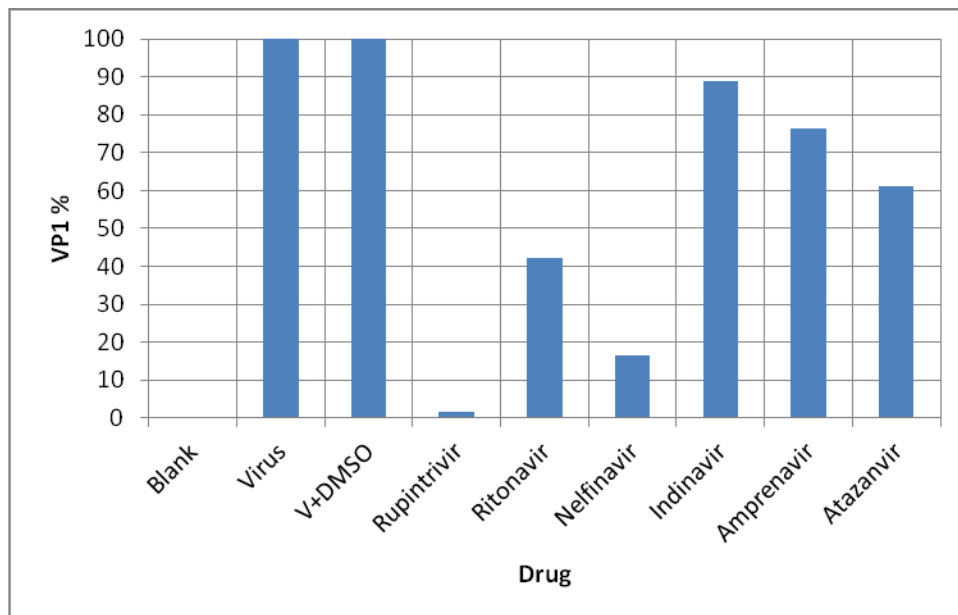
Source: Brick A & Wong CH, Org.Biomol.Chem.,2003,vol.1, 5-14 ;

<http://www.fda.gov/ForConsumers/byAudience/ForPatientAdvocates/HIVandAIDSActivities/ucm118915.htm>

Figure.6: Preliminary assay of HIV-1 Protease inhibitors with EV71 BrCr strain

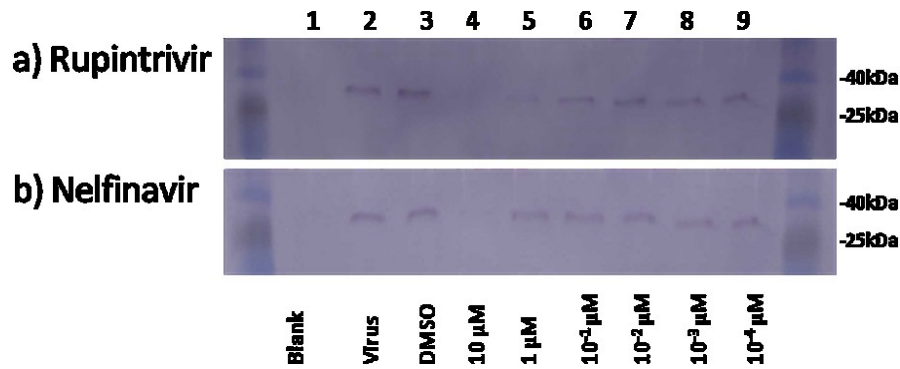


(a) EV71 (BrCr) VP1(~37KDa) bands detected by the western blot after treatment with HIV-1 protease inhibitors (concentration= 10 μ M).

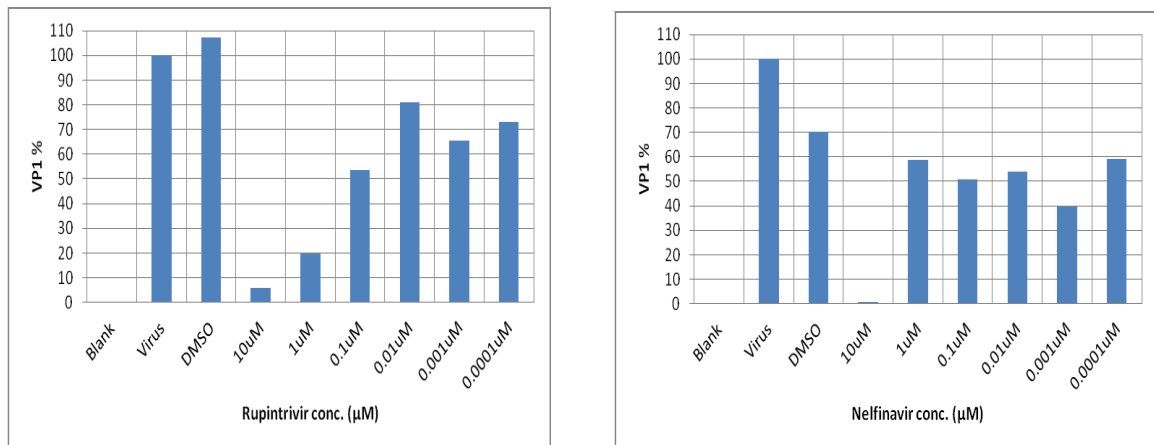


(b) EV71 (BrCr) VP1 protein quantified western blot yield obtained after treatment with HIV-1 protease inhibitors (concentration= 10 μ M).

Figure.7: Dose dependent screening of Nelfinavir with EV71 BrCr strain

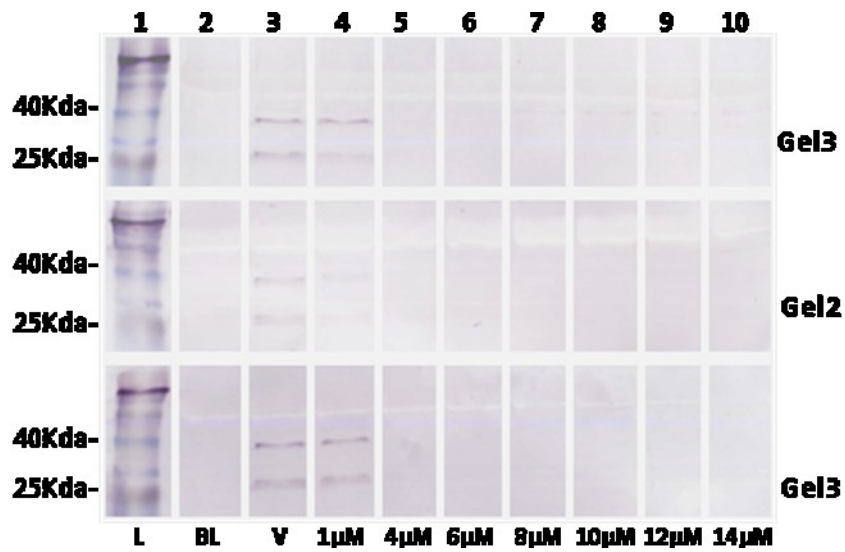


(a) Comparison of EV71 (BrCr) VP1(~37KDa) bands detected by the western blot after treatment with rupintrivir and nelfinavir

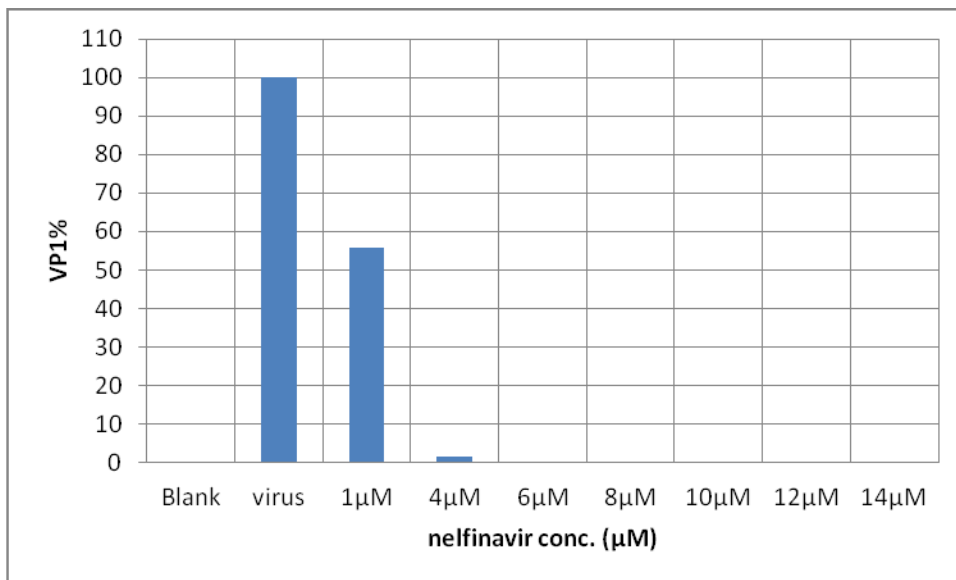


(b) Comparison of EV71 (BrCr) VP1 protein quantified western blot yield obtained after treatment with rupintrivir and nelfinavir

Figure.8: Effective concentration of Nelfinavir with EV71 BrCr strain

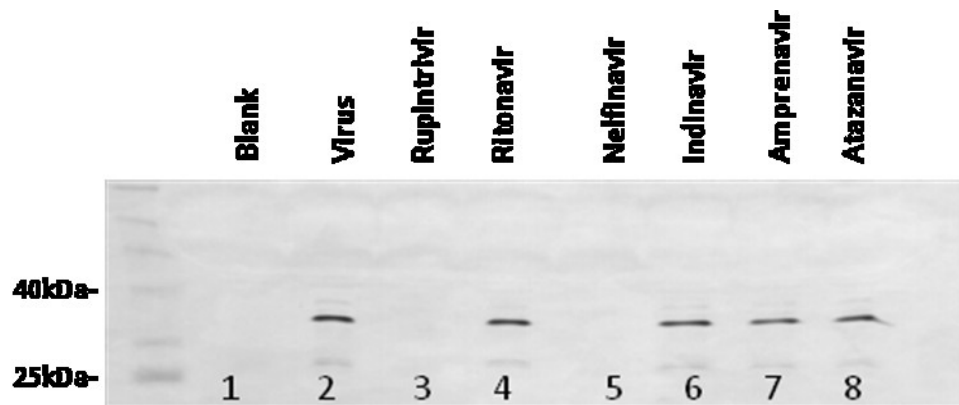


(a) EV71 (BrCr) VP1 (~37KDa) bands detected by the western blot after treatment with nelfinavir at varying concentrations

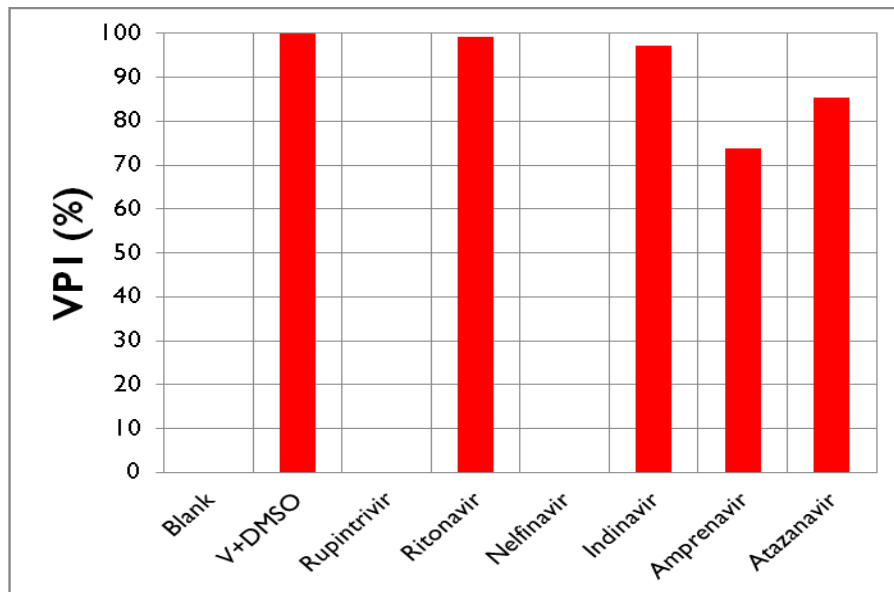


(b) EV71 (BrCr) VP1 protein quantified western blot yield obtained after treatment with nelfinavir at varying concentrations

Figure.9: Preliminary assay of HIV-1 Protease inhibitors with EV71 H strain

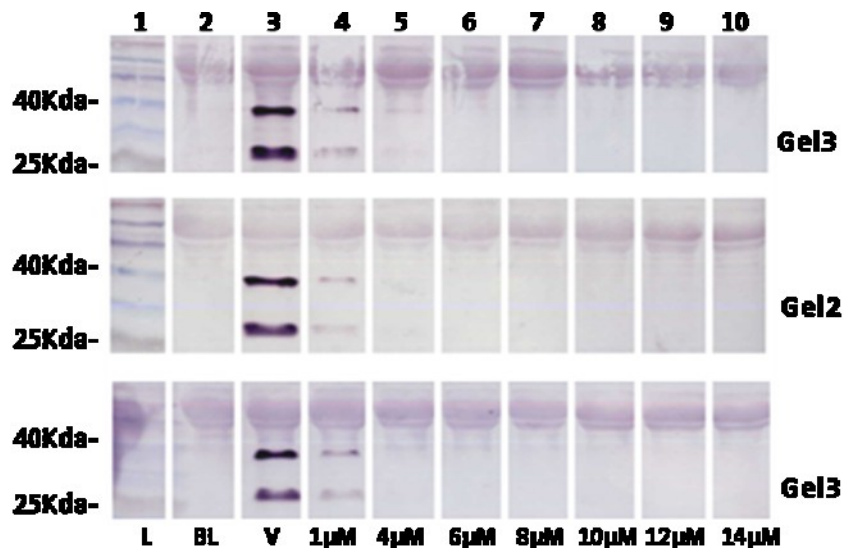


(a) EV71 (H strain) VP1(~37KDa) bands detected by the western blot after treatment with HIV-1 protease inhibitors (concentration= 10 μ M)

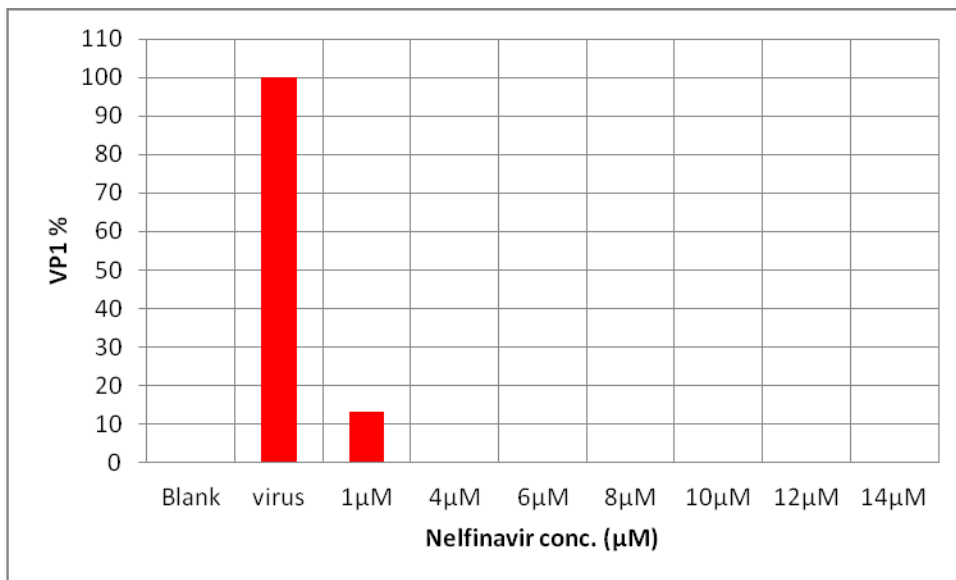


(b) EV71 (H strain) VP1 protein quantified western blot yield obtained after treatment with HIV-1 protease inhibitors (concentration= 10 μ M)

Figure.10: Effective concentration of Nelfinavir with EV71 H strain

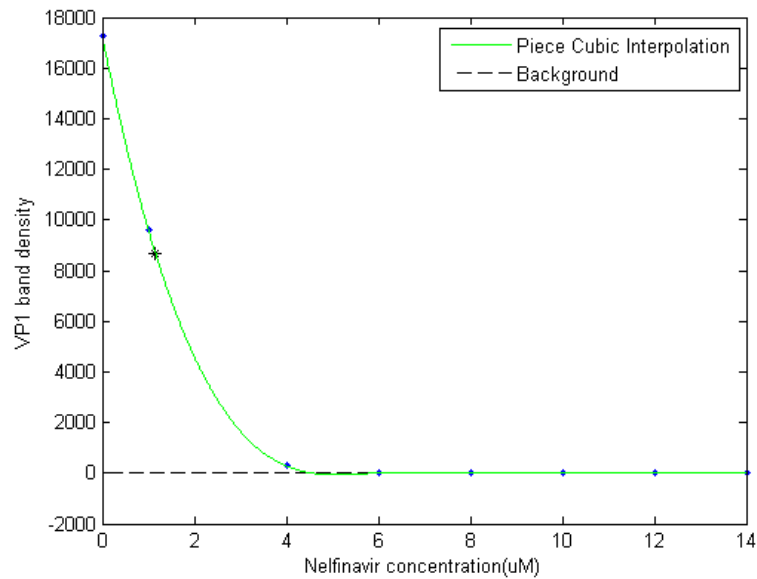


(a) EV71 (H strain) VP1 (~37KDa) bands detected by the western blot after treatment with nelfinavir at varying concentrations

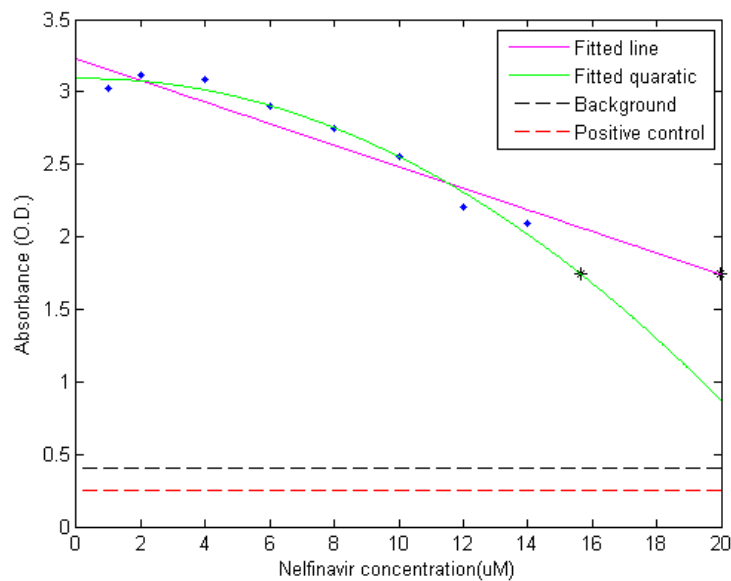


(b) EV71 (H strain) VP1 protein quantified western blot yield obtained after treatment with nelfinavir at varying concentrations

Figure.11: Nelfinavir cytotoxicity and effective concentration determination

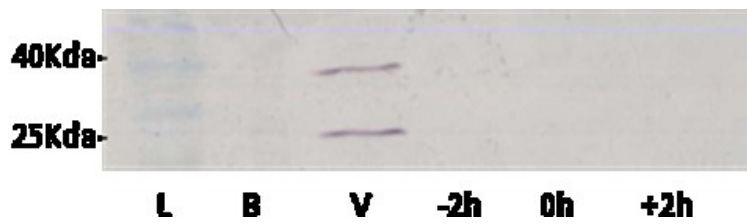


(a) EC₅₀ determination using piece-wise cubic polynomial fitting. * denotes the EC₅₀ estimated from the model.

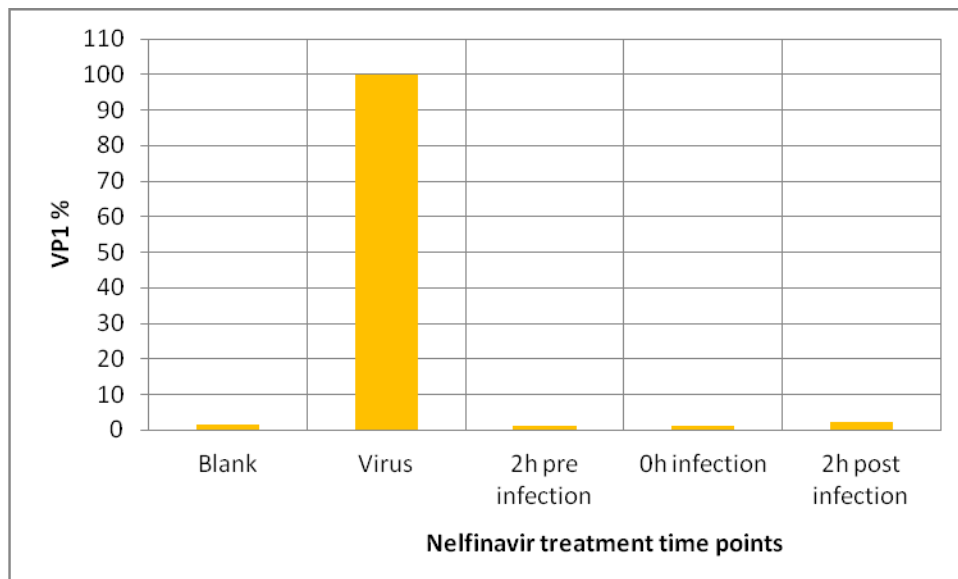


(b) CC₅₀ determination using linear regression and quadratic regression. * denotes the CC₅₀ estimated for each model.

Figure.12: Time of addition assay

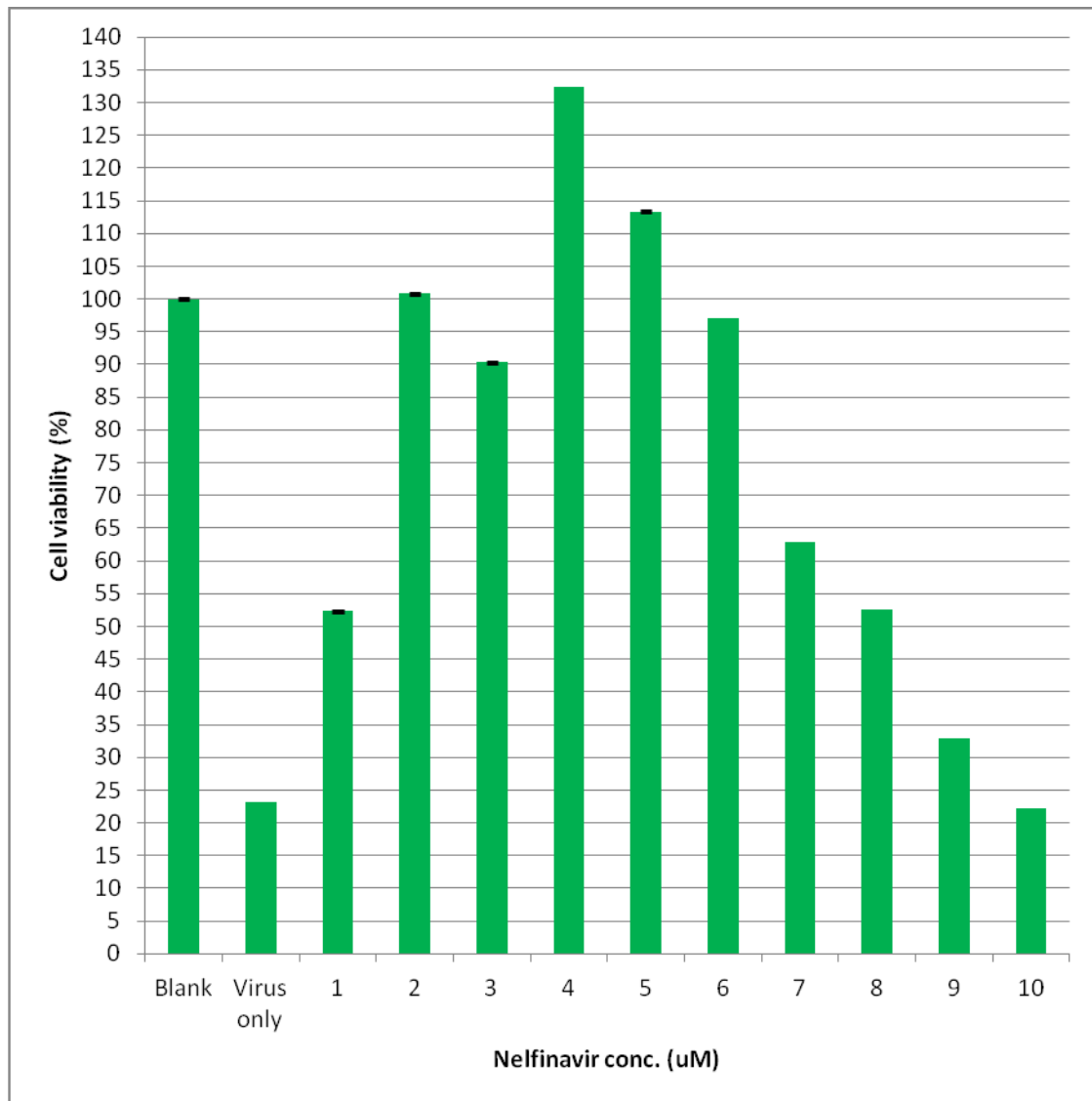


(a) VP1 (~37KDa) bands detected by the western blot after treatment with nelfinavir at different times: (-2h) indicates 2 hours pre treatment with drug, (0h) indicates concurrent addition of drug and virus and (+2h) indicates 2 hours delayed treatment with drug.

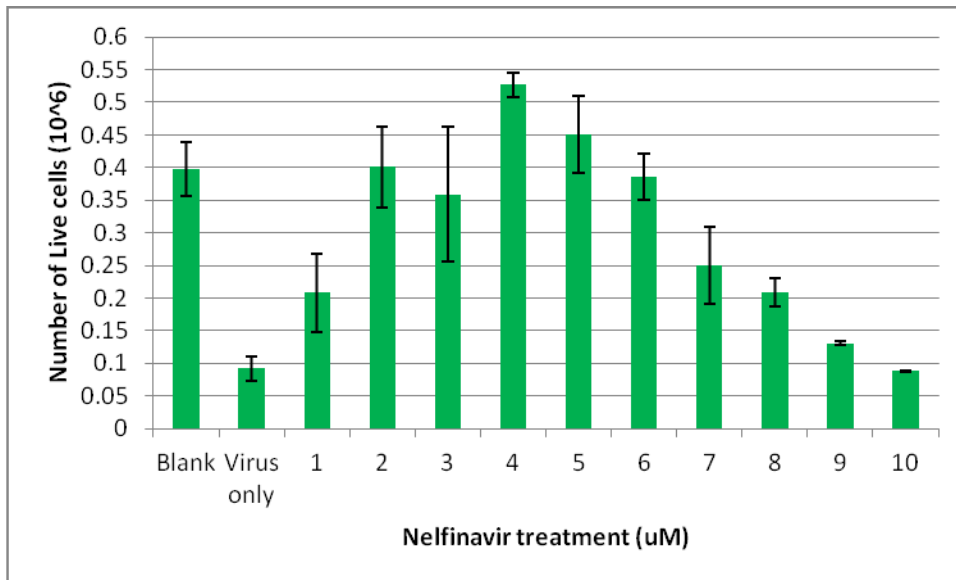


(b) VP1 protein quantified western blot yield obtained after treatment with nelfinavir at different times of the virus infection

Figure.13: Effective concentration of nelfinavir against hepatitis A virus

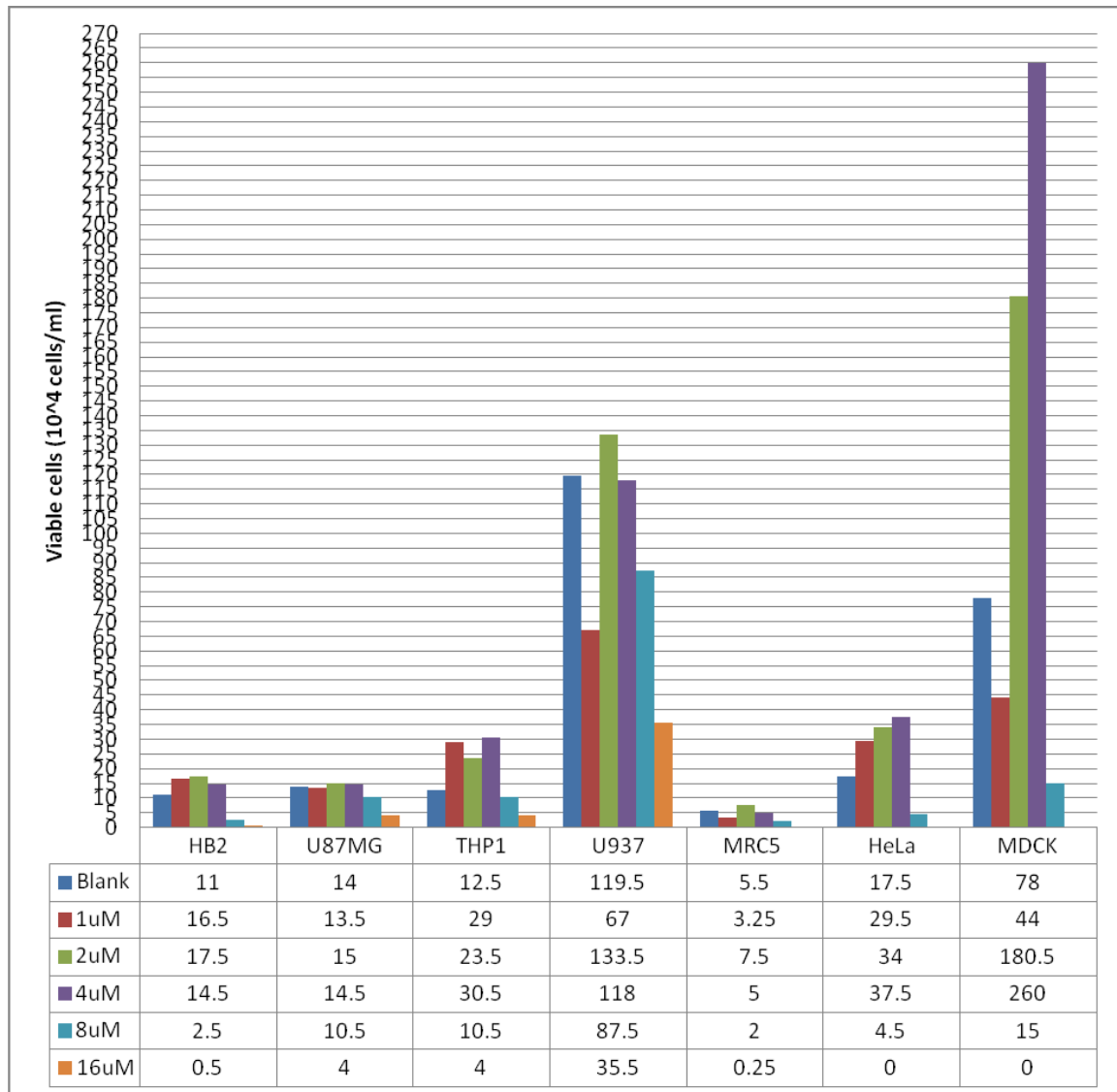


(a) Cell viability (%) obtained after treatment of HAV infected cells with nelfinavir at varying concentrations.

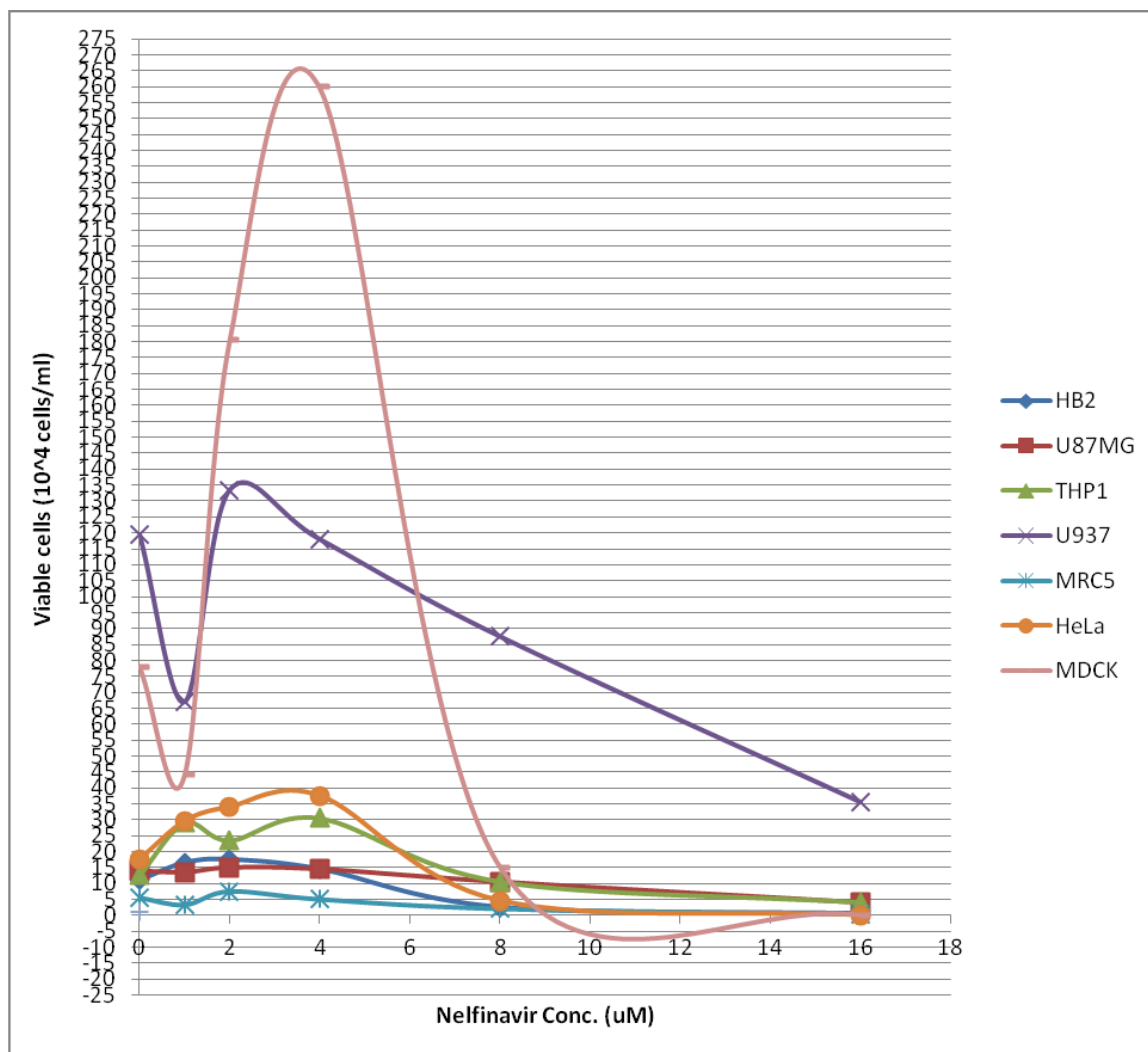


(b) Total live cells (in millions) counted after treatment of HAV infected cells with nelfinavir at varying concentrations.

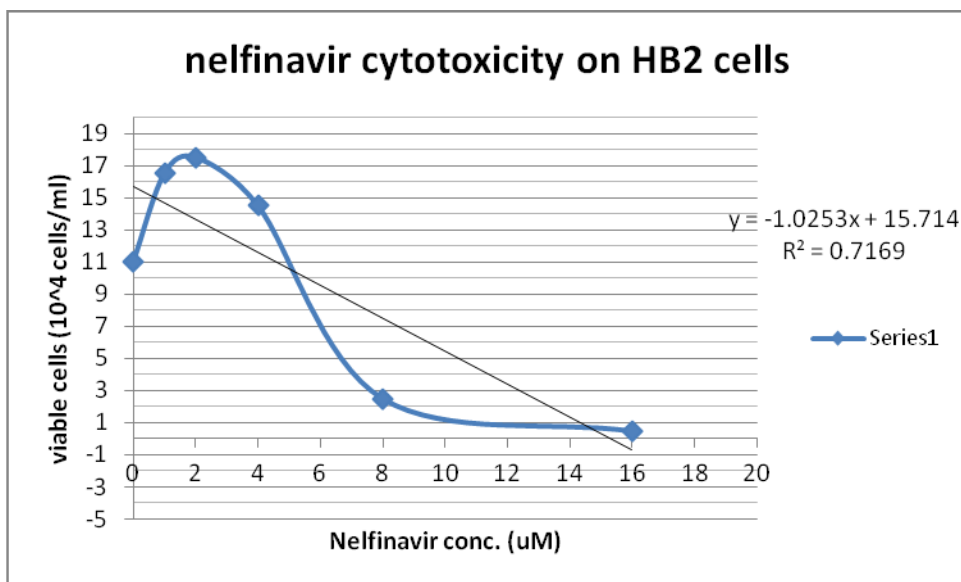
Figure.14: Nelfinavir cytotoxicity evaluation of different cell lines



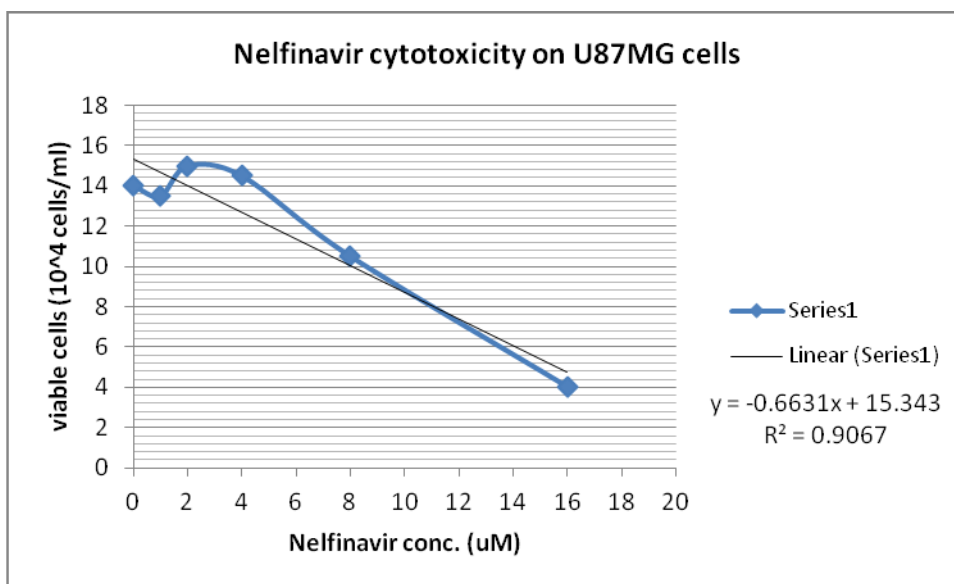
(a) Dose-effect of nelfinavir on viability of different cell types.



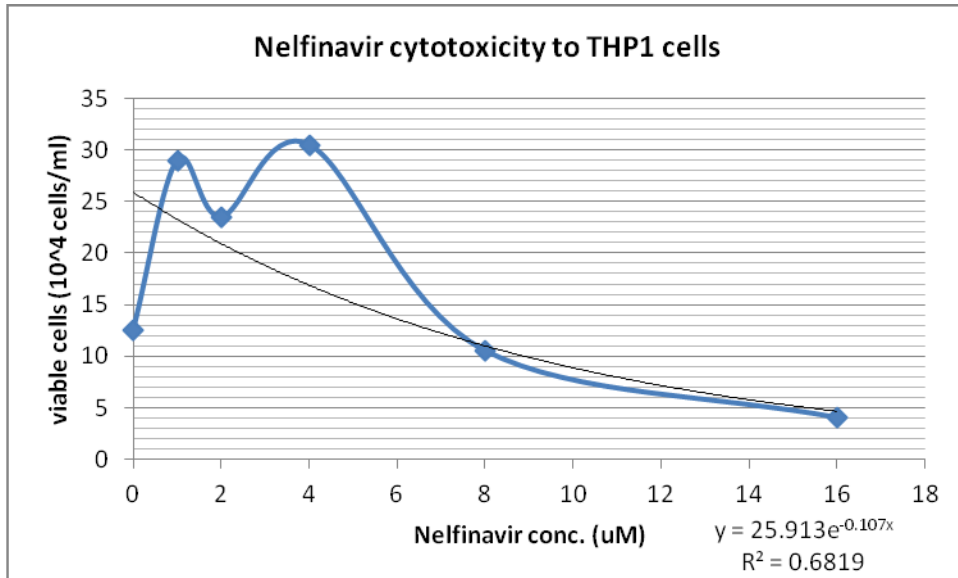
(b) Dose effect of nelfinavir on viability of different cell types.



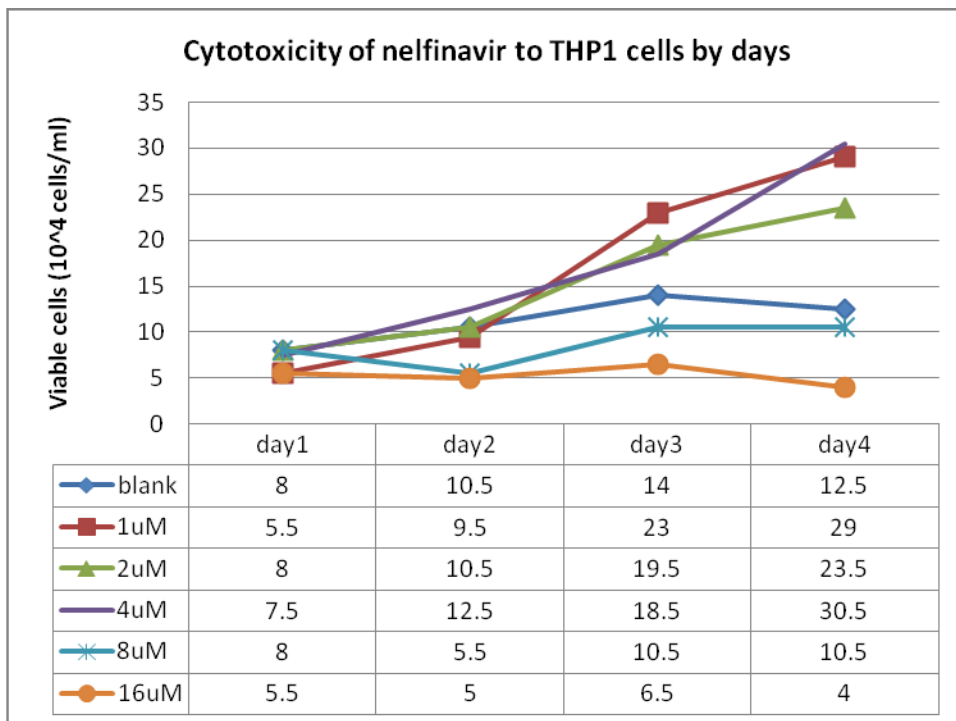
(c) Effect of varying nelfinavir concentration on HB2 cell line and the corresponding cell viability trend observed. Black line represents cell viability trend. R^2 = root mean square error



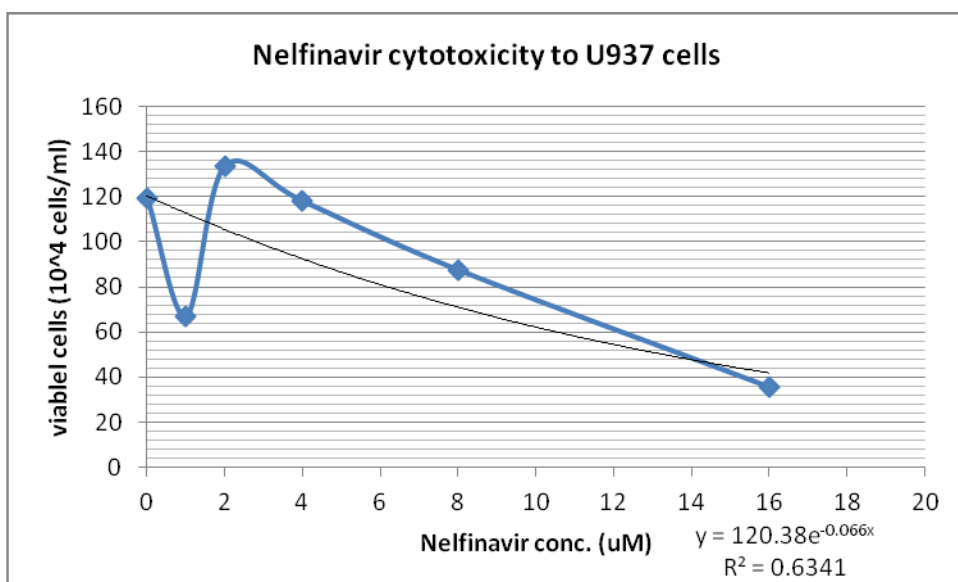
(d) Variation of U87MG cell viability with increasing concentration of nelfinavir. Black line represents cell viability trend. R^2 = root mean square error



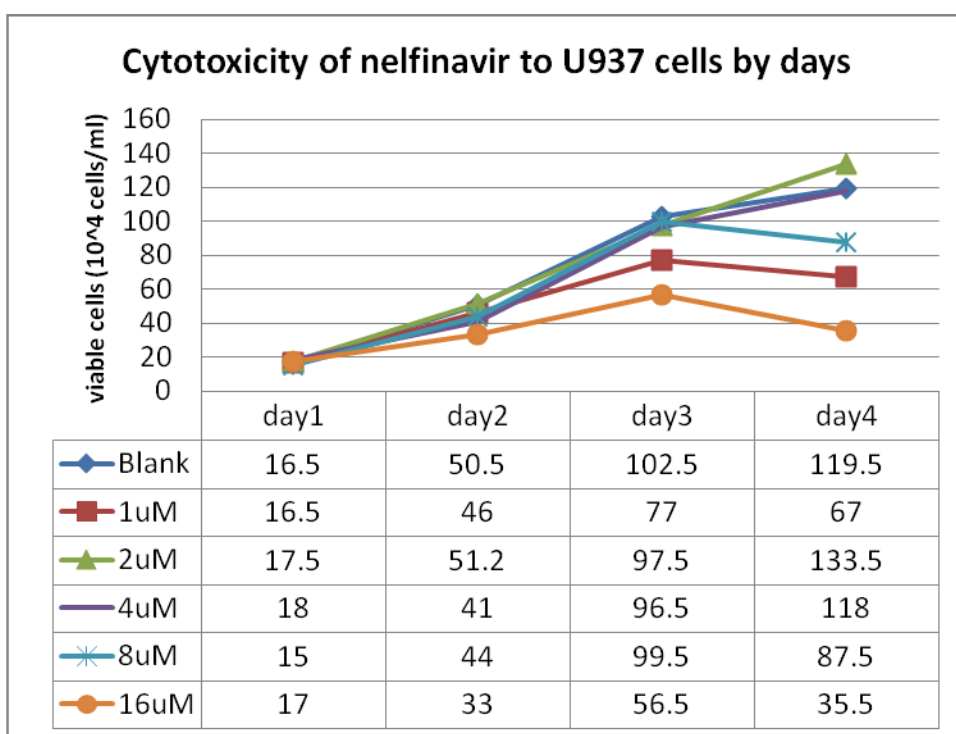
(e.1) Effect of nelfinavir concentration on THP1 cell line and the cell viability trend observed. R^2 = root mean square error



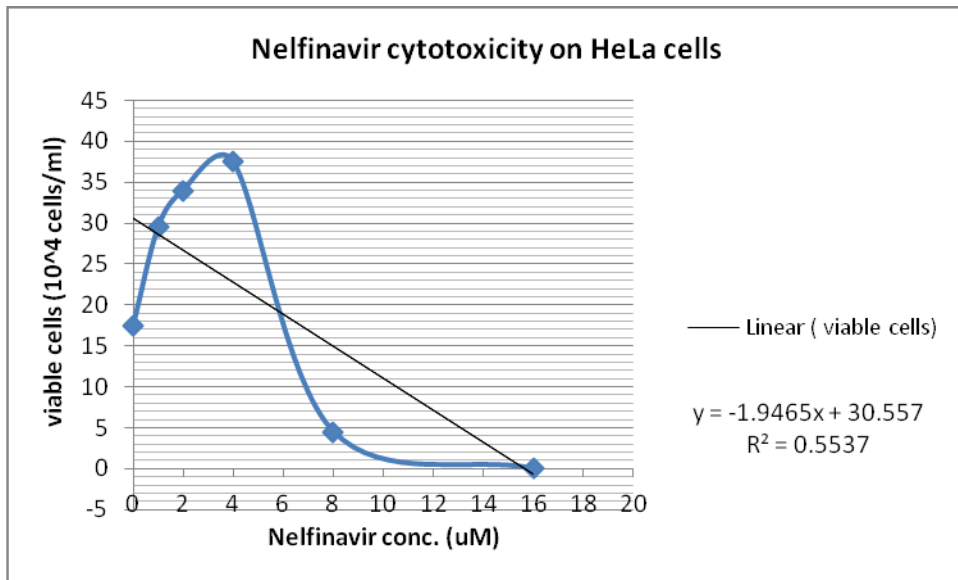
(e.2) Effect of nelfinavir concentration on THP1 cell viability observed over a period of 4 days.



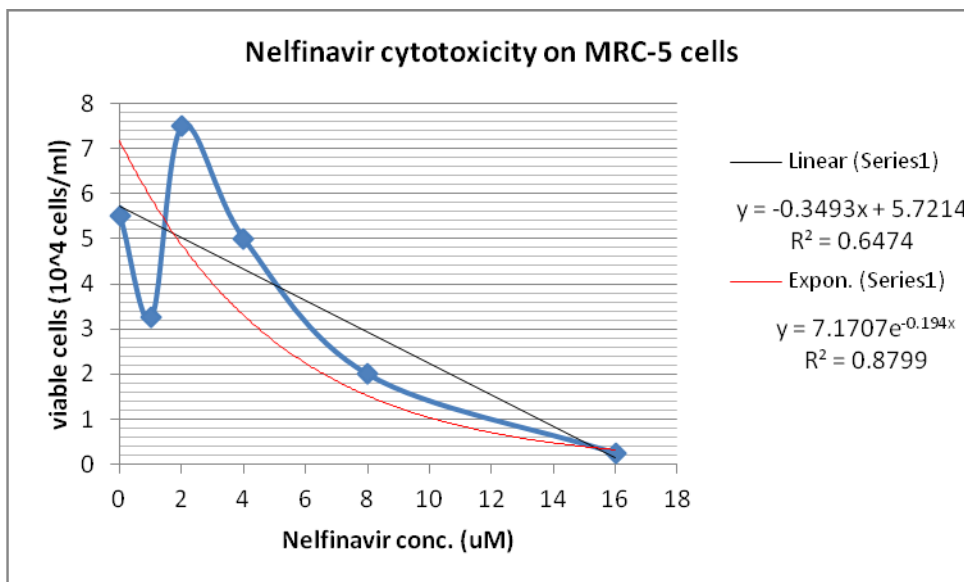
(f.1) Variation in U937 cell viability with increasing concentration of nelfinavir. cell viability trend is represented by the black line. R^2 = root mean square error



(f.2) Effect of nelfinavir concentration on U937 cell viability observed over a period of 4 days.

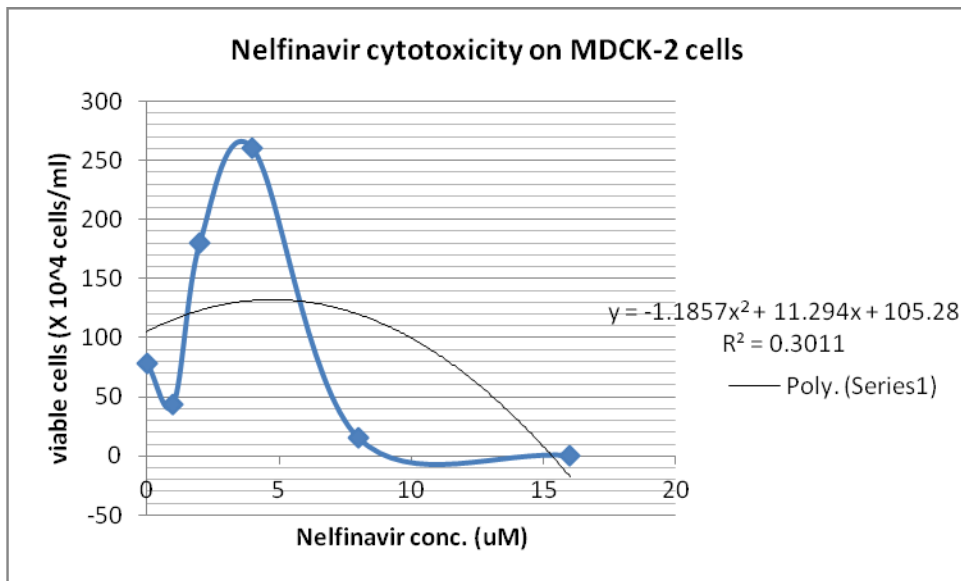


(g) Effect of nelfinavir concentration on HeLa cell line and corresponding the cell viability trend observed. R^2 = root mean square error



(h) Effect of nelfinavir concentration on MRC-5 cell line. Cell viability trend is represented by two lines. The red line represents the declining trend predicted using

exponential curve fitting. The black line represents the trend predicted using linear curve fitting. R^2 = root mean square error



(i) Dose wise effect of neflinavir on MDCK-2 cell line and the cell viability trend

observed. R^2 = root mean square error

Figure.15: Summary : clinical trials investigating nelfinavir's anti-cancer properties

S.N	CLINICAL TRIAL NUMBER, (PHASE)	TARGETED CANCER	STUDY ARM	NELFINAVIR DOSE	STUDY POPULATION	OUTCOME
1	NCT01485731 (I)	Cervical cancer	Nelfinavir+ Cisplatin+ Pelvic Radiation Therapy	875mg BID for 7 days	Primary, previously untreated, Histologically confirmed invasive carcinoma of uterine cervix. Clinical stages IIA, IIB, IIIA, IIIB, IVA.	Recruiting
2	NCT01445106 (I)	Solid Tumors	Nelfinavir	1250 mg PO BID; 6 dose increased till MTD	Histologically confirmed solid malignancy	Completed
3	NCT00704600 (I/II)	Colo-rectal cancer	Nelfinavir	Phase I: 6 tablets till MTD. Phase II: MTD	Histologically proven adenocarcinoma of the rectum	Active, not recruiting
4	NCT02080416 (0)	Gamma Herpesvirus-Related Tumors	Nelfinavir	3000 mg twice daily for 14 days	Non-Hodgkin Lymphoma Hodgkin Lymphoma Kaposi Sarcoma, Gastric Cancer, Nasopharyngeal Cancer EBV, Castleman Disease	Not yet recruiting
5	NCT01068327 (I)	Pancreatic cancer	Stereotactic Radiation Therapy+ Nelfinavir+ Gemcitabine Hydrochloride+ Leucovorin Calcium + Fluorouracil	Not specified	Pathologically confirmed adenocarcinoma of the pancreas	Active, not yet recruiting
6	NCT01079286 (I)	Renal cell cancer	Nelfinavir + temsirolimus dose escalation	250mg twice daily Dose escalation	histological or cytological confirmed malignancies	Completed
7	NCT01959672 (II)	Locally Advanced Pancreatic Cancer	Chemotherapy Oregovomab+ Stereotactic Radiotherapy +Nelfinavir	Dose not specified Duration: PO BID for 5 weeks	Pathologically confirmed adenocarcinoma of the pancreas, Recurrent Pancreatic Cancer Stage IA, IB, IIA, IIIB	Recruiting
8	NCT00436735 (I)	Metastatic, Refractory or Recurrent Solid	Nelfinavir	Dose not specified Duration: twice daily on days 1-	Histologically confirmed solid malignancy: Colorectal cancer	Unknown

		Tumors		21	Gastrointestinal Carcinoid Tumor, Head & Neck Cancer, Islet Cell Tumor Lung Cancer Metastatic Cancer Skin Neuroendocrine Carcinoma, Ovarian Cancer, Pheochromocytoma Sarcoma	
9	NCT00589056 (I/II)	Stage III Non-Small Cell Lung Cancer	Nelfinavir, Radiation Therapy, Cisplatin, & Etoposide	Dose not specified Duration: twice daily beginning 1-2 weeks	Histologically confirmed non-small cell lung cancer	Unknown
10	NCT01065844 (II)	Head, Neck neoplasms	Nelfinavir	1250 mg twice daily Duration: 7 days	Histological diagnosis of adenoid cystic carcinoma.	Active, not recruiting
11	NCT02024009 (II,III)	Localized Pancreatic Cancer - 2	GEMABX cape+Nelfinavir +50.4Gy in 28#	Not specified	Histologically or cytologically proven carcinoma of the pancreas	Not yet recruiting
12	NCT01164709 (I)	Advanced Hematologic Malignancies	bortezomib + Nelfinavir	625, 1250, 1875, or 2500 mg, 2x/d; PO days 1- 21	advanced hematologic malignancies	Completed
13	NCT01086332 (I/II)	Pancreatic Cancer	Gemcitabine + Nelfinavir	1250 mg twice daily	Pancreatic Neoplasms	Active, not recruiting
14	NCT01447589 (I)	Lung cancer	Nelfinavir + radical radiotherapy	Dose not specified tablets BD 7 days per week	Histologically confirmed NSCLC except bronchoalveolar cancer	Withdrawn
15	NCT01108666	Inoperable Stage III Non Small Cell Lung Cancer	Nelfinavir+ proton beam radiation+ chemotherapy	625 & 1250 mg PO BID.	Histologically confirmed diagnosis of NSCLC Stage IIIA or IIIB	Recruiting
16	NCT00791336 (II)	Non-small Cell Lung Cancer	Nelfinavir + Chemo-radiation	1250 mg twice daily	Histological diagnosis of non-small cell lung cancer that is Stage III (T1-3, pN2, M0) NSCLC	Terminated
17	NCT00915694 (I)	Glioblastoma Multiforme	Nelfinavir+ temozolomide	Dose not specified twice daily beginning 7-10	Histologically confirmed WHO grade IV supratentorial	Unknown

				days	astrocytoma (glioblastoma multiforme)	
18	NCT01925378 (II)	Cervical Intraepithelial Neoplasia	Nelfinavir	1,250 mg twice PO daily	Histologically proven CIN 2/3 or CIN 3	Recruiting
19	NCT01020292 (I)	Glioma	Nelfinavir	625, and 1250 mg BID tested	Histologically confirmed supratentorial WHO Grade IV astrocytoma	Active, not recruiting
20	NCT01555281 (I/II)	Progressive multiple myeloma	Nelfinavir and Lenalidomide/ Dexamethasone	1250mg twice daily	Multiple myeloma having progressed after at least two months of lenalidomide- containing therapy	Recruiting
21	NCT00233948 (I/II)	Recurrent, Metastatic, or Unresectable Liposarcoma	Nelfinavir	Dose not specified Duration: twice daily on days 1-28	Histologically confirmed liposarcoma, which is recurrent, metastatic or unresectable	Completed
22	NCT01728779 (II)	Oligo- metastases	Nelfinavir w/St ereotactic Body Radiation Therapy	1250 mg twice daily for 14 days	Tumor malignancies	Recruiting
23	NCT00003008 (II)	AIDS-Related Kaposi's Sarcoma	Nelfinavir	Not Specified	Histologically confirmed Kaposi's sarcoma requiring chemotherapy and/or currently being treated with paclitaxel	Completed
24	NCT00002185 (II)	Cutaneous & Mucosal KS in AIDS Patients	Nelfinavir	Not Specified	Diagnosed KS proven by biopsy	Completed
25	NCT00694837 (I/II)	Glioblastoma	Nelfinavir	1000mg BID	Histologically confirmed glioblastoma multiforme at primary diagnosis	Suspended

Sravya Kurapati

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EDUCATION

Johns Hopkins Bloomberg School of Public Health

(Expected May 2014)

Science Masters (ScM) in Molecular Microbiology & Immunology

VIT University, Vellore, India

(June 2006 - May 2010)

Bachelor of Technology (B.Tech) in Biotechnology

RELEVANT COURSE WORK

Immunology-I & II, Neuroimmunology, Parasitology, Malariology, Fundamentals of Virology, Vector Biology, Food & Water Borne Diseases, Public Health Biology, Infectious Diseases & Child Survival, Molecular Biology, Clinical & Epidemiological aspects of tropical diseases, Introduction to Biomedical Sciences

LABORATORY SKILLS

Viral & Tissue Culture, Real Time PCR, Matrix Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometer, Flowcytometry, ELISpot, High Performance Liquid Chromatography, Western Blot, Cloning

RESEARCH EXPERIENCE

BASIC SCIENCE

- **Johns Hopkins Bloomberg School of Public Health, Baltimore**

[Dr. Xiao-Fang Yu's Laboratory](#)

(Mar 2013 - present)

- As a *graduate student*, evaluating the potential antiviral effect of HIV protease inhibitors against *Enterovirus 71* infection in vitro. Also determining the effective dose for different strains of the virus along with assessing the cytotoxicity profile of the drug. Currently, testing the broad spectrum antiviral effect of the drug on other picornaviruses

- **All India Institute of Medical Sciences, New Delhi**

[Dr. Madhu Vajpayee's Laboratory](#)

(Dec 2009 - July 2012)

- As a Junior Research Fellow worked on identifying T-cell epitopes associated with control of viral replication in Indian & South African HIV- 1 infected individuals
- As a *Research Trainee* examined the effect of HIV-1 on T-cell activation, differentiation & exhaustion^{1,2,3}
- Also, conducted comparative analysis of Real Time RT PCR Assay and Reverse Transcriptase ELISA for quantization of HIV-1 viral load in subtype C patients⁴

[Dr. J.C Samantaray's Laboratory](#)

(Aug 2010 - Nov 2010)

- As a *Research Trainee* Investigated use of MALDI TOF-MS as a novel and rapid diagnostic tool for *Mycobacterium tuberculosis* clinical isolates⁵

PUBLIC HEALTH

- **All India Institute of Medical Sciences, New Delhi**
Dr. Madhu Vajpayee's Laboratory (Dec 2009 - July 2012)
 - Illustrated correlation between CD4 counts, HIV-1 viral load and socio-demographic profile of HIV-1 infected patients attending a tertiary care center in India
 - Highlighted the presence of HIV-1 infection in adolescents in India ⁶
 - Studied the presence of HIV infection and associated risk factors in women attending a Voluntary Counseling & Testing Center in North India ⁷
 - Performed external quality assurance of HIV samples for surveillance studies; assisted in establishing Quality System Essentials at the National HIV Reference Laboratory
- **Agilent technologies Pvt. Ltd & Agastya Foundation, Bengaluru** (May 2009 - June 2009)
 - Led the 'WE-HEAL' community health project in investigating water and food contamination in 8 study villages of Karnataka & Andhra Pradesh each (India). Levels exceeding the permissible limits of heavy metals, nitrates, total dissolved solids and microbial contaminants were found in the food and water samples. Study findings and sustainable solutions were explained to villagers

PUBLICATIONS

1. Mojumdar K, Vajpayee M, Chauhan NK, Singh A, Singh R, **Kurapati S** (2012). Altered T cell differentiation associated with loss of CD27 and CD28 in HIV infected Indian individuals. *Cytometry B Clinical Cytometry*
2. Mojumdar K, Vajpayee M, Chauhan NK, Singh A, Singh R, **Kurapati S** (2011). Loss of CD127 & increased immunosenescence of T cell subsets in HIV infected individuals. *Indian Journal of Medical Research*
3. Vajpayee M, Negi N, **Kurapati S** (2013). The Enduring Tale of T cells in HIV Immunopathogenesis. *Indian Journal of Medical Research*
4. Kokkayil P, **Kurapati S**, Negi N, Vajpayee M (2014). Comparative evaluation of a reverse transcriptase based assay for HIV-1 viral load quantitation in resource limited settings. *Journal of Virological Methods*
5. Panda A, **Kurapati S** et al (2013). Rapid identification of clinical mycobacterial isolates by protein profiling using matrix assisted laser desorption ionization-time of flight mass spectrometry. *Indian Journal of Medical Microbiology*
6. **Kurapati S** et al (2012). Adolescents Living with HIV: An Indian Profile. *AIDS Research and Treatment*
7. Negi N, **Kurapati S** et al. Women Living with HIV – A Glimpse from India. Under Review in *Indian Journal of Medical Microbiology*

PRESENTATIONS

- **Posters**
 - Evaluation of protective efficacy of HIV Protease Inhibitors against EV71 infection. *Molecular Microbiology & Immunology Retreat, Johns Hopkins* (2013)
 - 1st Prize. Rapid and novel identification of clinical mycobacterial isolates using MALDI-TOF MS in routine clinical laboratory. *Indian Association of Medical Microbiologists* (Delhi Chapter) Academic Meet (2010)

- **Abstracts**

- T-cell differentiation and immunosenescence defects in HIV infected individuals in India. *Keystone Symposia on HIV Evolution, Genomics and Pathogenesis* (2011)
- Muffled Voices: Adolescents living with HIV in India. *6th International AIDS Society Conference on HIV Pathogenesis, Treatment and Prevention* (2011)
- Study of CD4+CD8+ double positive T lymphocyte activation & exhaustion in HIV-1 infected Indian patients. *6th International AIDS Society Conference on HIV Pathogenesis, Treatment & Prevention* (2011)

VOLUNTARY COMMUNITY SERVICE

- 7 day National Service Scheme Camp in Solavaram village, Tamil Nadu, India (2009)
- Voluntary Blood Donation Camp. Youth Red Cross, India (2008)

PROFESSIONAL DEVELOPMENT

Computer skills: Microsoft Office Suite, Prism GraphPad 5

Languages: English, Hindi, Telugu, German

EXTRA CURRICULAR ACTIVITIES

- Co-President. South Asian Graduate Association, Johns Hopkins (2013-14)
- Organizing Committee member for SENNET'07 & '09, international conference on sensors & networking
- Press & Media coordinator for GRAVITAS'09, a tech-management fest (2009)
- Secretary & Editor. Indian Society for Technical Education (ISTE) students' chapter at VIT university (2006-07 & 08)

PERSONAL DETAILS

Born, November 26, 1988; enjoy reading, drawing, travelling and hiking